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BIOCHEMICAL AND PHYSIOLOGICAL STUDIES ON PLANT OXYLIPINS

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Anthony Louis Schilmiller

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BIOCHEMICAL AND PHYSIOLOGICAL STUDIES ON PLANT OXYLIPINS

By

Anthony Louis Schilmiller

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ABSTRACT

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By

Anthony Louis Schilmiller

Oxylipins comprise a diverse group of biologically active molecules that arise from oxidative metabolism of polyunsaturated fatty acids, and have been implicated in regulating diverse aspects of plant growth and development. Most studies of plant oxylipins have focused on the hormone jasmonic acid (JA), which is a key regulator of plant responses to insect attack, and recently has been implicated as a mobile signal for activation of long distance defense responses.

The synthesis of oxylipins begins with lipoxygenase (LOX) catalyzed production of hydroperoxy fatty acids, which are subsequently metabolized by the CYP74 family of cytochromes P450. Different CYP74 enzymes direct the hydroperoxides into different oxylipin biosynthetic routes. Part of this dissertation focuses on the characterization of a novel *CYP74* gene that was identified by searching the tomato expressed sequence tag database. Biochemical analysis of the recombinant enzyme showed that it is an allene oxide synthase (LeAOS3) that metabolizes both 9- and 13-hydroperoxides of linoleic and linolenic acid. However, the increased specificity of LeAOS3 for 9-hydroperoxides indicated that this enzyme functions in the 9-LOX pathway in tomato. *LeAOS3* expression in roots was shown to be highly inducible by JA-treatment and wounding. cDNA microarray experiments were performed to identify genes that are co-regulated with LeAOS3 in JA-treated roots. In contrast to roots, expression of *LeAOS3* in

hypocotyls of germinating seedlings was constitutive and independent of JA. Immunolocalization of LeAOS3 in roots and hypocotyl showed expression in cortex cell layers. The dual modes of LeAOS3 regulation are discussed in the context of the function of the 9-LOX pathway.

Pioneering studies in the 1980s demonstrated the involvement of β-oxidation in JA biosynthesis. Until now, however, no genes encoding β-oxidative enzymes that are required for the production of JA have been identified. The second half of this dissertation focuses on the role in JA biosynthesis of acyl-CoA oxidases (ACX), which catalyze the first step of β-oxidation. Forward genetic analysis in tomato indicated that a specific ACX isoform (ACX1A) was required for JA biosynthesis. Biochemical analysis of recombinant ACX1A showed that this isoform has the catalytic capacity to oxidize CoA-esters of JA biosynthetic intermediates. *Arabidopsis thaliana* mutants that are defective in the orthologs of *ACX1A* were isolated and used to study the role of ACX in herbivore and pathogen defense. These results showed that JA is required for defense against chewing insects, whereas cyclic intermediates are sufficient for defense against a necrotrophic pathogen. The characterized *Arabidopsis* ACX mutants will be useful for future studies aimed at further dissection of the signaling properties of JA and its cyclic intermediates.

ACKNOWLEDGEMENTS

When I visited MSU to interview for grad school, my flight was delayed and got into Lansing sometime around 2:00 AM in the morning. But there was Dr. Gregg Howe at the Lansing airport ready to give me a ride to the hotel. From the very beginning and over the years as a graduate student, Gregg has been a great advisor and mentor, and I would like to thank him for all his guidance and support. I truly feel that I made the best decision in joining his lab, and I look forward to continue collaborating with Gregg during my time as a post-doc in Dr. Robert Last's lab here at MSU.

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Lastly I must thank my family who has supported me in my many (at least according to them) years as a student. I only wish MSU was a little closer to home so I could go plow a field or split some wood to relieve the stress sometimes.

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Chapter 1

Introduction: Oxylipin Biosynthesis and Function

Plants are constantly subjected to a variety of environmental insults including drought, disease-causing pathogens, UV radiation, temperature stress, herbivory, and mechanical wounding. Nevertheless, plants have evolved sophisticated ways to protect themselves against these environmental hostilities. Mechanisms for protection involve both constitutive and induced defenses. Constitutive defenses include morphological (e.g. thorns) and physical (e.g. cuticle layer) barriers that prevent damage from such stresses as herbivore attack or water loss during drought. Other constitutive defenses involve preformed chemicals or proteins ("phytoanticipins") that function to deter pest attack. Alternatively, induced defense strategies involve recognition of stress or damage followed by production of signals that activate the synthesis of defense compounds.

One of the first responses of plants to stress is altered lipid and fatty acid metabolism. A wealth of evidence indicates that oxidized lipids and fatty acids, collectively called oxylipins, play an important role in plant development and in plant responses to environmental cues (Howe and Schilmiller, 2002). In addition to their role as signals, oxylipins also function as defensive chemicals. These findings have fueled interest in understanding how and why oxylipins are synthesized in plants.

I. Oxylipin biosynthesis and function

Oxylipin biosynthesis begins with the addition of oxygen to polyunsaturated fatty acids. This step can be catalyzed by lipoxygenases or dioxygenases or can occur non-enzymatically by auto-oxidation (Hamberg et al., 1999; Feussner and Wasternack, 2002; Mueller, 2004). The resulting hydroperoxy fatty acids undergo a

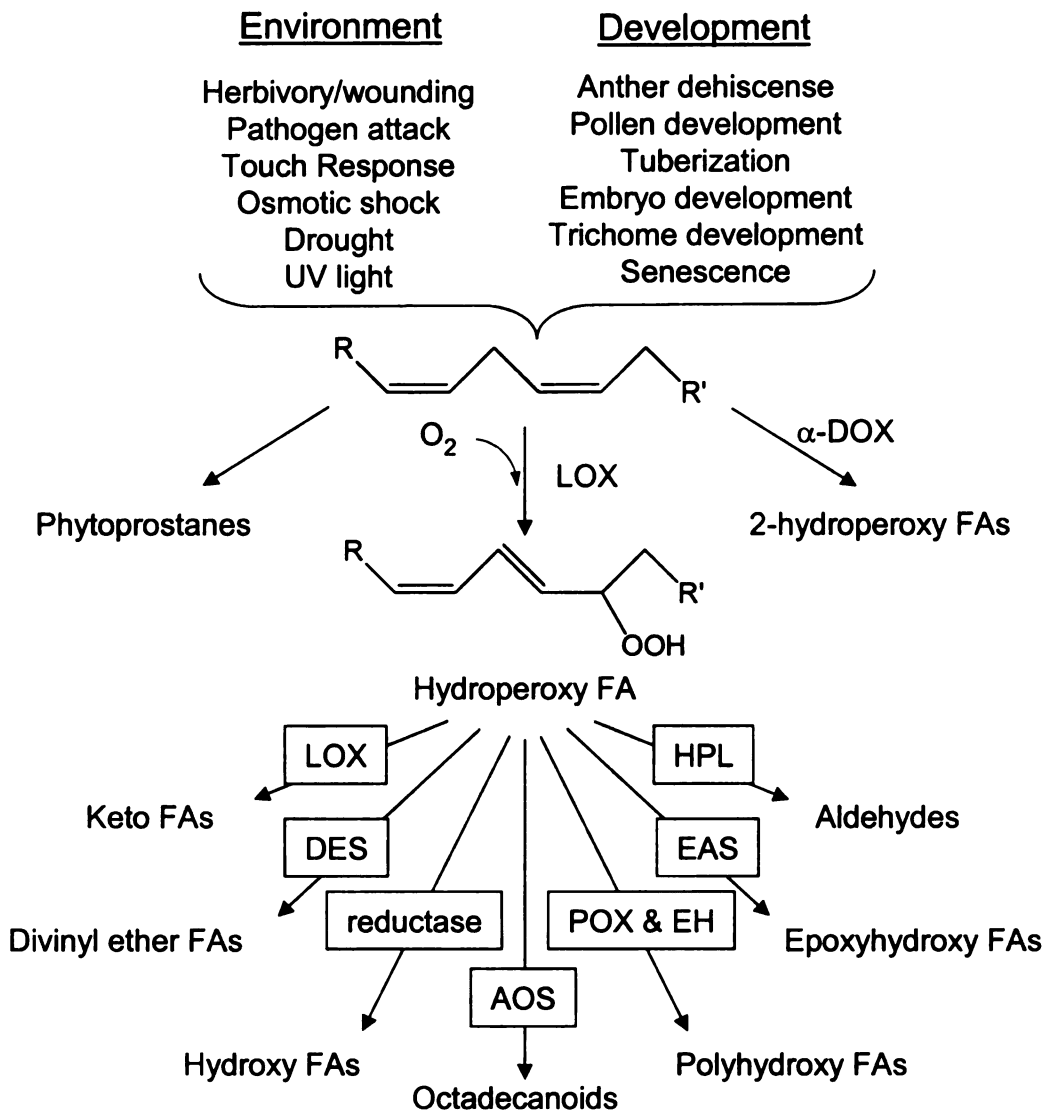

Figure 1.1 Biosynthesis of oxylipins in response to environmental and developmental cues. Many environmental and developmental signals can trigger LOX-catalyzed production of hydroperoxy fatty acids that are further acted upon by various enzymes to generate a wide range of oxylipins. LOX, lipoxygenase; α-DOX, α-dioxygenase; DES, divinyl ether synthase; AOS, allene oxide synthase; POX, peroxygenase; EH, epoxide hydrolase; EAS, epoxyalcohol synthase; HPL, hydroperoxide lyase (figure adapted from Howe and Schilmiller, 2002).

variety of cleavage and rearrangement reactions that give rise to the full suite of oxylipins (Figure 1.1). Oxylipins are formed in all higher plants and are also found in mosses (Dembitsky, 1993) and some species of marine alga (Gerwick, 1993). The similarity of plant oxylipins to eicosanoids in animals, which can function in immune responses and cell differentiation, demonstrates the importance of oxidative metabolism of lipids across kingdoms.

Lipoxygenase

Lipoxygenases (LOX) are non-heme, iron-containing di-oxygenases that add molecular oxygen to polyunsaturated fatty acids (PUFAs, i.e. linoleic and linolenic acids) to generate hydroperoxy fatty acids (Figure 1.2). LOXs are classified according to their positional specificity of linoleic acid oxygenation. Those isoforms that add a hydroperoxy group at the 9-position are named 9-LOXs, whereas those that add to the 13-position are called 13-LOXs. In

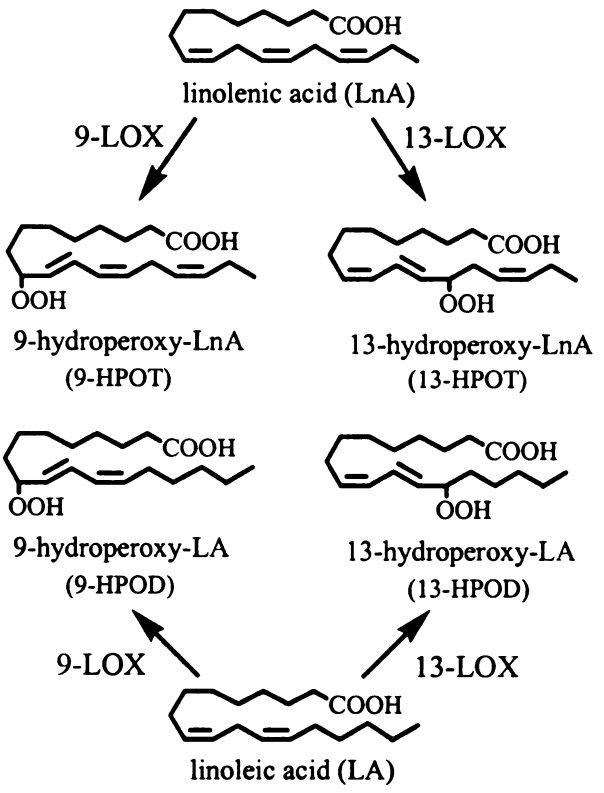

Figure 1.2 Lipoxygenase-catalyzed production of hydroperoxy fatty acids from linolenic and linoleic acids.

some plants, 16-carbon fatty acids can also serve as LOX substrates. A major focus of oxylipin research is to determine the physiological roles of the 9- and 13-LOX pathways. A useful approach has been to genetically alter expression of one or more *LOX* genes, and then study the effects on development and defense. Silencing of the *LOX-H1* gene of potato showed for the first time that a LOX could supply substrate specifically to the hydroperoxide lyase (HPL) branch of oxylipin biosynthesis (Leon et al., 2002). Antisense suppression of a *13-LOX* in tobacco caused reduction in wound-induced JA accumulation and resulted in increased herbivore performance on the LOX-deficient plants (Halitschke and Baldwin, 2003). Similar approaches have been used to study the role of the 9-LOX pathway. Expression of some *9-LOX* genes is induced upon treatment with fungal pathogens (Veronesi et al., 1996). In one case, suppression of a *9-LOX* gene in tobacco resulted in enhanced susceptibility to *Phytophthora parasitica* (Rance et al., 1998). Hydroperoxides derived from either the 9- and 13-LOX pathway have been shown to accumulate during the hypersensitive response of plants to pathogen infection. However, the specific profile of hydroperoxy fatty acids depends on the plant used in the study (Montillet et al., 2002). A role for 9-LOX in plant development has also been suggested based on the suppression of 9-LOX activity in potato, which caused fewer and deformed tubers (Kolomiets et al., 2001).

Other hydroperoxide-forming activites

In addition to oxylipins derived from hydroperoxides generated by LOXs, there are other routes for the synthesis of hydroperoxy fatty acids. In animals, cyclooxygenases (COX) catalyze the formation of endoperoxides that undergo further metabolism to give

rise to prostaglandins, which function in diverse aspects of development and stress responses (Smith et al., 2000). In plants, an enzymatic activity unrelated to LOX was found to catalyze the dioxygenation of fatty acids at the C-2 (α-) position. This enzyme was named α-dioxygenase (α-DOX; Figure 1.3) (Hamberg et al., 1999). Sequence analysis and comparison of the predicted structure of α-DOX revealed homology with the crystal structure of ovine COX (Sanz et al., 1998). 2-hydroperoxy fatty acids can be reduced to 2-hydroxy fatty acids, or undergo successive decarboxylation and oxidation reactions to shorten the fatty acid by one carbon. α-DOX was originally isolated by differential mRNA display in a search for genes induced by a pathogen elicitor (Sanz et al., 1998). Later studies showed that induction of α-DOX and increased production of 2-hydroxy fatty acids exert a tissue-protective effect in pathogen infected leaves (Hamberg et al., 2003).

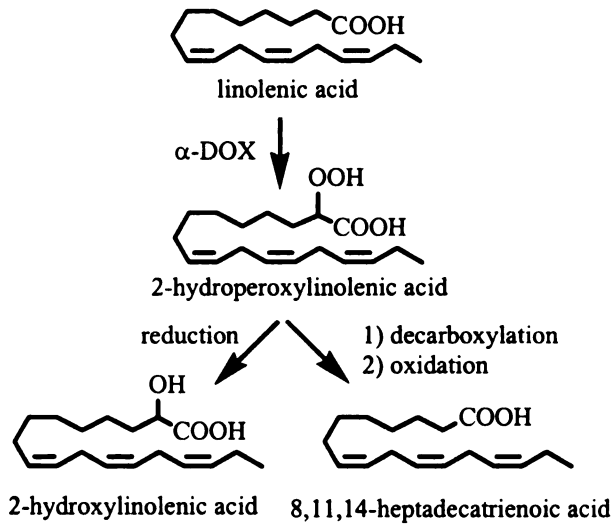

Figure 1.3 α-Dioxygenase pathway

Auto-oxidation of fatty acids catalyzed by free-radicals generates hydroperoxy fatty acids that are converted to prostaglandin-like compounds named phytoprostanes (Figure 1.4) (Krischke et al., 2003). The production of phytoprostanes was shown to

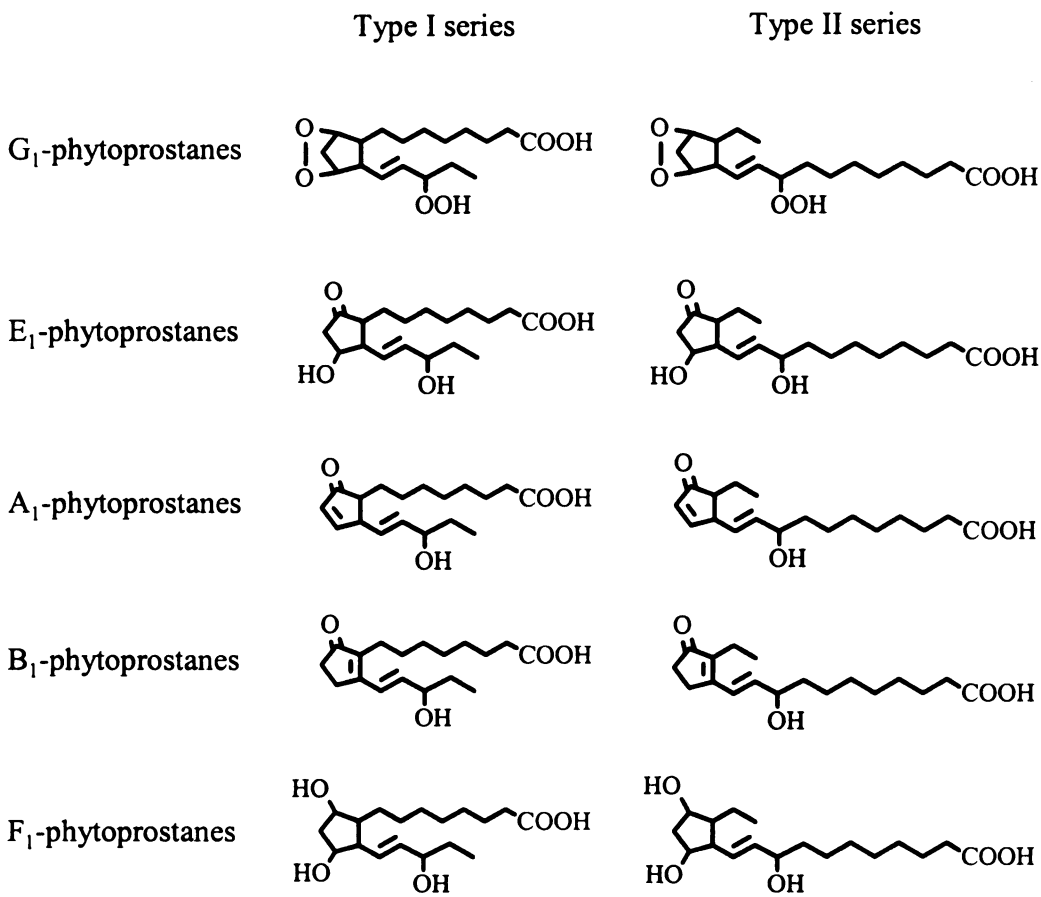

Figure 1.4 Type I and Type II phytoprostanes synthesized from linolenic acid. Non-enzymatic oxygenation of linolenic acid, which is catalyzed by free-radicals, results in the production of two series of racemic phytoprostanes.

increase in tobacco cell cultures under peroxide stress and tomato leaves infected by the necrotrophic fungus *Botrytis cinerea*. Application of *in vitro* synthesized phytoprostanes to tobacco cell cultures resulted in the induction of phytoalexin synthesis, suggesting that phytoprostanes act as signals to elicit responses during oxidative stress (Thoma et al., 2003).

Hydroperoxide-metabolizing enzymes

Lipoxygenase

Whereas the primary reaction catalyzed by LOX is the peroxidation of fatty acids, LOXs also convert hydroperoxy fatty acids to fatty acid ketodienes (KODE) and ketotrienes (KOTE) from dienoic and trienoic fatty acids, respectively (Figure 1.5) (Kuhn et al., 1991). Until recently, relatively little was known about the function of KODEs and KOTEs in plants. Wounded and pathogen infected leaves were found to accumulate high levels of KODEs and KOTEs compared to untreated controls (Vollenweider et al., 2000). Moreover, infiltration of *Arabidopsis* leaves with KODE or KOTE induced the expression of the *glutathione-S-transferase* gene and caused cellular damage. These

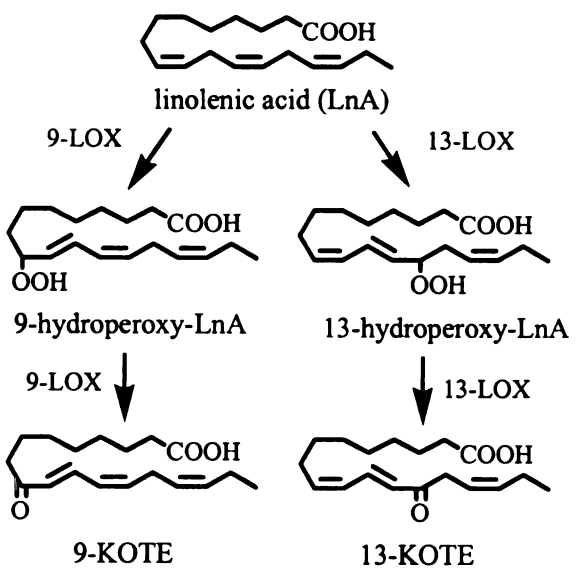

Figure 1.5 LOX-catalyzed production of ketodienes

effects of KODE and KOTE on plant physiology were attributed to the electrophilic properties of the α,β-unsaturated carbonyl feature of the molecules (Almeras et al., 2003). A recently identified LOX from the moss *Physcomitrella patens* was found to produce not only hydroperoxy and keto fatty acids, but was also found to possess a fatty-acid lyase activity (Senger et al., 2005).

Epoxy alcohol synthase

Epoxy alcohol synthase (EAS) converts fatty acid hydroperoxides into epoxy alcohol fatty acids (Figure 1.6) (Hamberg, 1999). The EAS products are then metabolized by epoxide hydrolases to give trihydroxy fatty acids. Currently, genes encoding EAS have not been identified. Oxylipin profiling experiments have shown that 9-HPOD-

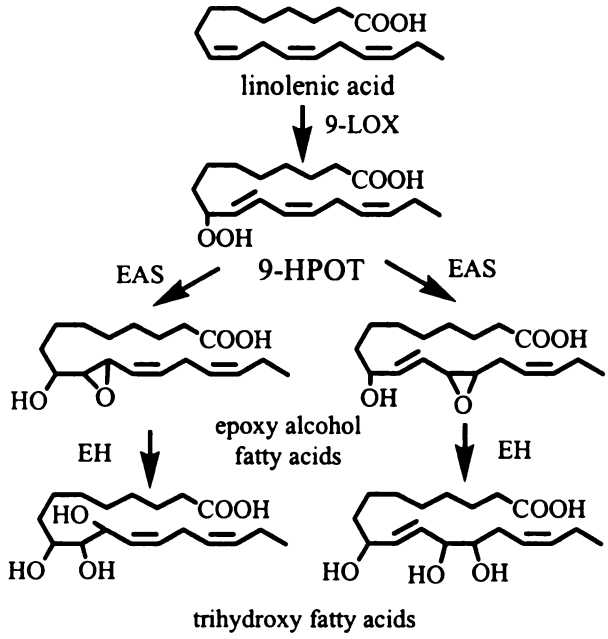

Figure 1.6 Epoxy alcohol synthase-catalyzed oxylipins.

(EAS, epoxy alcohol synthase; EH, epoxide hydrolase)

derived trihydroxy fatty acids from the EAS pathway are induced in potato leaves upon pathogen infection (Gobel et al., 2002). These results, together with the observation that trihydroxy fatty acids inhibit the growth of pathogenic fungi (Masui et al., 1989), suggest a role for the EAS pathway in defense against plant pathogens.

Peroxygenase

The peroxygenase (POX) pathway catalyzes the production of trihydroxy fatty acids that are regiochemically identical to products of the EAS pathway. However, POX-derived trihydroxy fatty acids differ from those of the EAS pathway with respect to their stereochemistry. POX is a membrane-bound, heme-containing oxidase distinct from cytochrome P450 enzymes (Blee, 1998). Like EAS, no gene has been identified that encodes POX. The production of trihydroxy fatty acids by the POX pathway suggests a role in pathogen defense. More recently POX was shown to catalyze the epoxidation step for the synthesis of C18-monomers for cutin biosynthesis (Lequeu et al., 2003).

Reductase

In the reductase pathway, hydroperoxy fatty acids are reduced to hydroxy fatty acids by an unknown mechanism. The synthesis of 13-hydroxy fatty acids via the reductase pathway was shown to be induced upon treatment with salicylic acid (SA) (Weichert et al., 1999). This induction, and the ability of 13-hydroxy-octadecatrienoic acid to induce expression of the *PR1b* gene, suggests a role for the reductase pathway in establishment of SA-mediated defense. Another study implicated hydroxy fatty acids as

intermediates in the degradation of storage lipids in germinating seedlings (Feussner et al., 1997).

CYP74 cytochromes P450

Of the enzymes that metabolize hydroperoxy fatty acids, the CYP74 family of cytochromes P450 is the best characterized. CYP74 enzymes make up a divergent family of P450s with catalytic properties distinct from typical P450 monooxygenases (Howe and Schilmiller, 2002). Whereas P450 monooxygenases require O_2 and a NADPH-dependent P450 reductase for activity, CYP74 family members do not. Rather, the hydroperoxide substrate of CYP74s serves as the oxygen donor and a source of reducing equivalents. CYP74 P450s also exhibit a reduced affinity for carbon monoxide. Interestingly, these catalytic features of plant CYP74 P450s are shared by prostacyclin synthase and thromboxane synthase, two P450s involved in the synthesis of eicosanoids (Hecker and Ullrich, 1989). In plants, there are three types of CYP74 enzymes: hydroperoxide lyase (HPL), divinyl ether synthase (DES), and allene oxide synthase (AOS). Based on amino acid sequence similarity, CYP74s are divided into four subfamilies. CYP74A consists of AOSs, whereas CYP74B consists of HPLs that metabolize 13-hydroperoxy fatty acids. CYP74C includes AOSs and HPLs that have the capacity to metabolize both 9- and 13-hydroperoxy fatty acids. CYP74D is made up of DESs that metabolize 9-hydroperoxy fatty acids.

Hydroperoxide lyase

HPL catalyzes the cleavage of hydroperoxy fatty acids to aliphatic aldehydes and ω-keto-fatty acids (Figure 1.7). The volatile aldehydes and their corresponding alcohols contribute to the so-called "fresh green" odor of fruits and vegetables, and have generated interest due to their use as food additives to restore the freshness of foods after sterilization processes (Noordermeer et al., 2001). HPL was first described in 1973 in a study of banana volatiles (Tressl and Drawert, 1973). The first gene encoding HPL was later cloned from bell pepper, and demonstrated that the enzyme is a CYP74 cytochrome P450 (Matsui et al., 1996). To date, *HPL* genes have been isolated and characterized from a variety of plants including *Arabidopsis*, guava, alfalfa, cucumber, and tomato (Bate et

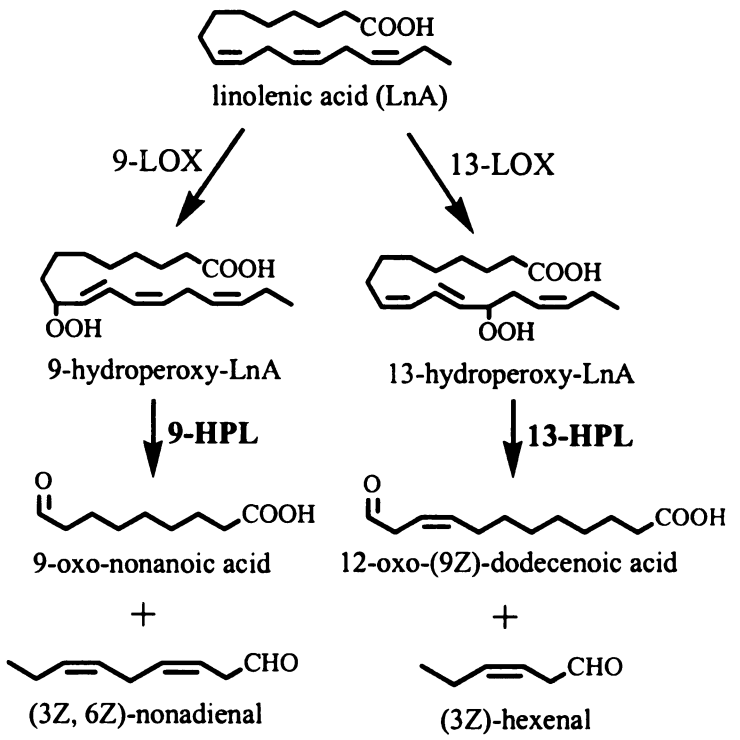

Figure 1.7 Hydroperoxide lyase-catalyzed cleavage of fatty acid hydroperoxides.

al., 1998; Howe et al., 2000; Matsui et al., 2000; Noordermeer et al., 2000; Tijeta et al., 2000). While many HPLs show specificity for 13-hydroperoxy fatty acids, some were found to cleave both 9- and 13-hydroperoxides (Matsui et al., 2000; Tijet et al., 2001). Localization experiments showed that 13-HPL, which lacks a transit peptide, is targeted to the outer membrane of the chloroplast envelope (Froehlich et al., 2001).

Products of the HPL pathway not only contribute to the aroma of plant tissues, but possess other physiological functions. The C12 product arising from cleavage of 13-hydroperoxy linolenic acid is the precursor of the wound hormone traumatin that has been implicated in wound healing (Zimmerman and Coudron, 1979). C6-aldehydes from the 13-HPL pathway induce a subset of wound-responsive genes, suggesting that HPL products act as signals in plant defense (Bate and Rothstein, 1998). The HPL-derived aldehydes have also been shown to have antimicrobial effects and reduce aphid fecundity *in vitro* (Deng et al., 1993). More recently, antisense-meditated suppression of *13-HPL* in potato was shown to cause an increase in aphid performance. This finding indicated a role for the HPL pathway in defense against sucking insects that feed on phloem contents (Vancanneyt et al., 2001). More work is needed to identify physiological functions of the 9-HPL pathway.

Divinyl ether synthase

Divinyl ether fatty acids are synthesized from polyunsaturated fatty acids via the sequential action of LOX and DES (Figure 1.8). In 1972, Galliard and Phillips demonstrated the synthesis of divinyl ethers in cell-free extracts from potato tubers, and suggested 9-hydroperoxy fatty acids as an intermediate (Galliard and Phillips, 1972).

Later work demonstrated the same activity in tomato roots (Caldelari and Farmer, 1998). Homogenates of meadow buttercup leaves also possess a DES activity that acts on 13-hydroperoxides of linoleic acid (Hamberg, 1998). Some species of marine algae can also synthesize divinyl ether fatty acids from 18- and 20-carbon fatty acids (Proteau and Gerwick, 1993; Jaing and Gerwick, 1997). The first *DES* gene was isolated from tomato, and shown to encode a CYP74D cytochrome P450 (Itoh and Howe, 2001).

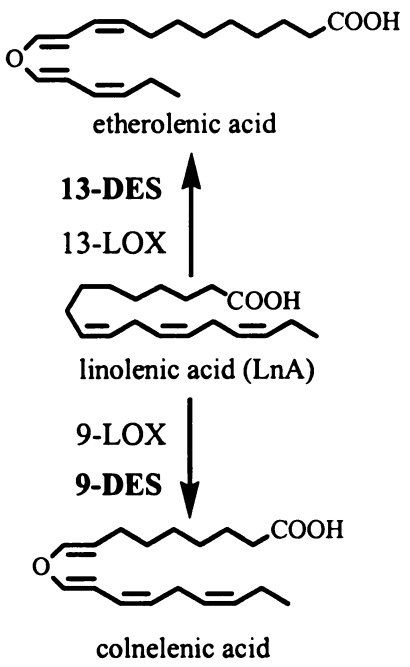

Figure 1.8 Divinyl ether synthase

The physiological function of the DES pathway in plants is not known. However, a role for divinyl ether fatty acids in defense against plant pathogens has been suggested by a number of studies. Oxylipin profiling experiments showed that elicitors and pathogen treatments led to an increase in the accumulation of the divinyl ethers colneleic (CA) and colnelenic acid (CnA), which are produced from the 9-hydroperoxides of linoleic and linolenic acid, respectively (Weber et al., 1999; Gobel et al., 2001; Gobel et al., 2002). Treatment of *Phytophthora infestans* with purified CA and CnA resulted in decreased mycelial growth and inhibition of cytospore germination, suggesting that divinyl ether fatty acids could function in limiting the growth of this pathogen (Weber et al., 1999). Expression of the potato *DES* gene was also shown to be induced by pathogen treatment (Stumpe et al., 2001).

Allene oxide synthase

Over thirty years ago, it was shown that an enzyme activity in flaxseed extracts converts 13-hydroperoxy linoleic acid (13-HPOD) to 13-hydroxy-12-oxo-9(Z)-octadecenoic acid (α-ketol). This enzyme was named "hydroperoxide isomerase" (Zimmerman, 1966; Zimmerman and Vick, 1970). Later experiments demonstrated the conversion involved formation of an unstable allene oxide that undergoes spontaneous hydrolysis to form α- and γ-ketols or a cyclopentenone compound (Figure 1.9). This finding prompted re-naming of the enzyme to allene oxide synthase (AOS) (Brash et al., 1987; Hamberg, 1987). Purification of AOS from flaxseed showed the enzyme to be a cytochrome P450 (Song and Brash, 1991). The *AOS* gene cloned from flaxseed became the founding member of the CYP74 family of P450s (Song et al., 1993). *AOS* genes have since been isolated from several plants including *Arabidopsis*, tomato, tobacco, and barley (Laudert et al., 1996; Howe et al., 2000; Maucher et al., 2000; Sivasankar et al., 2000; Ziegler et al., 2001). Similar to 13-HPL, 13-AOS is located in the chloroplast envelope. Unlike 13-HPL, however, 13-AOS contains an N-terminal chloroplast targeting sequence and is located on the inner leaflet of the inner envelope membrane (Froehlich et al., 2001). Interestingly, *Plexaura homomalla* (Caribbean sea whip coral) AOS occurs as a fusion protein with LOX (Koljak et al., 1997). Although the AOS domain of the fusion protein catalyzes the production of allene oxide fatty acids, sequence and structural data indicate that this AOS is more similar to catalase, and is not a cytochrome P450 (Oldham et al., 2005).

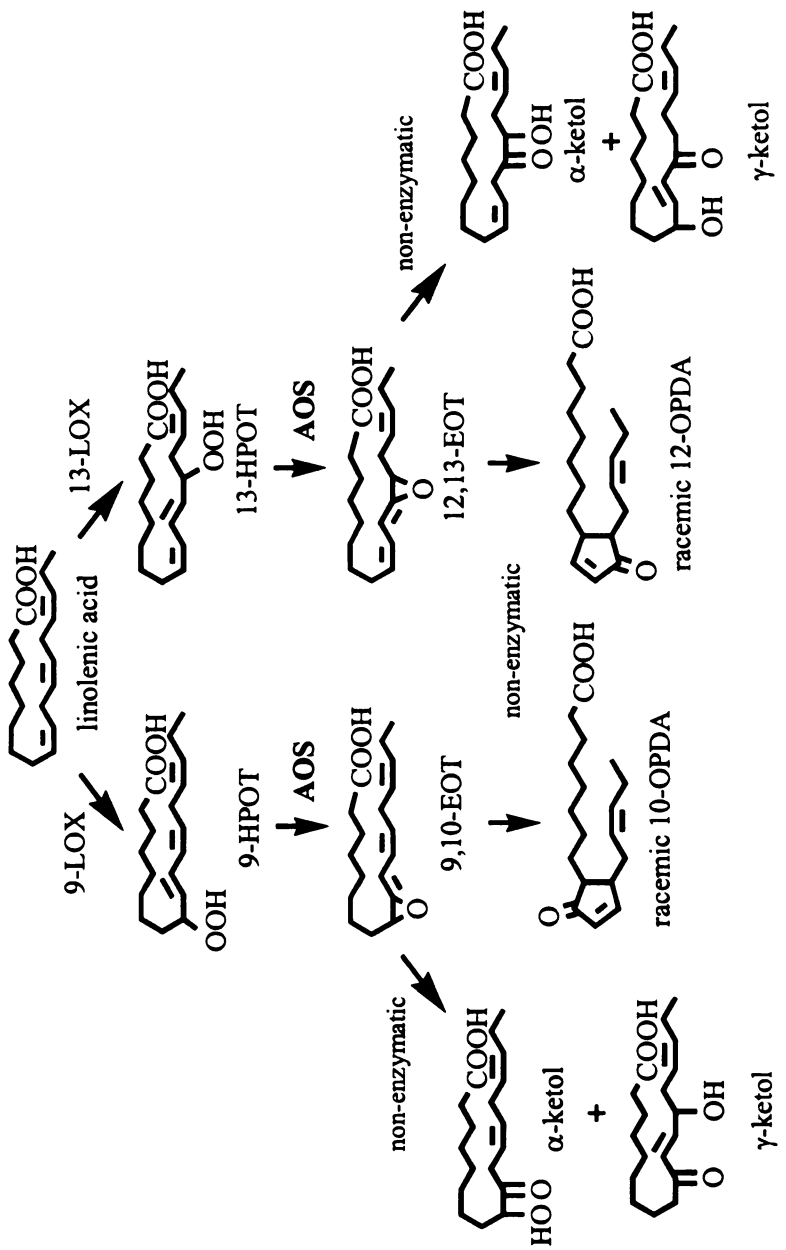

Figure 1.9 Allene oxide synthase-catalyzed reactions. 9,10-EOT, 9,10-epoxy-10,12,15-octadecatrienoic acid; 12,13-EOT, 12,13-epoxy-9,11,15-octadecatrienoic acid; OPDA, oxophytodienoic acid

More research on the AOS pathway in plants has been focused on 13-AOS because of its involvement in biosynthesis of jasmonic acid (JA). A knockout mutation in the *Arabidopsis AOS* gene impairs the synthesis of JA and causes male sterility (Park et al., 2002; von Malek et al., 2002). A more detailed discussion of the physiological roles of the 13-AOS pathway and JA is presented later in this chapter.

9-AOS activity has been reported in several plant species (Gardner, 1970; Graveland, 1973; Grechkin et al., 2000). However, only recently were cDNAs that encode 9-AOSs isolated (Maucher et al., 2000; Itoh et al., 2002). The tomato 9-AOS (LeAOS3), which is the subject of chapters 2 and 3 of this dissertation, represents the first AOS shown to function in the 9-LOX pathway. In contrast to the clear understanding of the 13-AOS pathway and its involvement in JA biosynthesis, virtually nothing is known about the physiological function of the 9-AOS pathway. Part of the focus of this dissertation was to study the regulation of *LeAOS3* in an effort to elucidate the role of the 9-AOS pathway in plants.

II. Jasmonate biosynthesis

Since the discoveries of MeJA in oils of jasmine (Demole et al., 1962) and JA in fungal culture filtrates (Aldridge et al., 1971), JA and related members of the jasmonate family (collectively referred to as JAs) have been the most extensively studied of all the plant oxylipins. The biosynthetic pathway for JA was elucidated by Vick and Zimmerman in the early 1980s (Vick and Zimmerman, 1983). They demonstrated that fatty acid hydroperoxides of linoleic acid undergo enzymatic cyclization to form 12-oxophytodienoic acid (12-OPDA). It is now recognized that the cyclization to 12-OPDA

is catalyzed by the sequential action of AOS and allene oxide cyclase (AOC). Vick and Zimmerman went on to show that 12-OPDA is further metabolized to JA through reduction of the cyclopentenone ring followed by three successive rounds of β-oxidation (Vick and Zimmerman, 1984). Work in the years following Vick and Zimmerman's contribution has identified most of the genes involved in JA biosynthesis (Schaller et al., 2004). This work has also demonstrated the importance of JAs in plant growth and development, and in regulating defense responses against herbivores and pathogens (Wasternack and Hause, 2002).

The synthesis of JA is initiated in the chloroplast by LOX-catalyzed addition of molecular oxygen to linolenic acid (Figure 1.10). The resulting 13-hydroperoxy-linolenic acid (13-HPOT) is acted upon by AOS to form the allene oxide 12,13-epoxy-9,11,15-octadecatrienoic acid. Allene oxide cyclase (AOC) converts the allene oxide to the first cyclic intermediate in the pathway, 12-OPDA. At this point, 12-OPDA is transported to the peroxisome where the cyclopentenone ring is reduced by OPDA reductase (OPR3) to give 3-oxo-2(2'-pentenyl)-cyclopentane-1-octanoic acid (OPC-8:0). OPC-8:0 is esterified to coenzyme A (CoA) and undergoes three successive rounds of β-oxidation followed by removal of CoA, presumably by a thioesterase, to yield JA.

Figure 1.10 The pathway for JA biosynthesis. JA synthesis begins in the chloroplast with conversion of linolenic acid to 12-OPDA by the sequential action of LOX, AOS, and AOC. 12-OPDA moves to peroxisome where it undergoes a reduction step followed by three rounds of β-oxidation to yield JA. JA modifications include methylation by JMT or conjugation to amino acids by JAR1. The perception of JAs resulting in changes in gene expression requires COI1, an F-box protein that is part of a E3-ubiquitin ligase complex. (JMT, JA methyltransferase; JME, JA methyl esterase; JAR1, jasmonate response1; COI1, coronatine insensitive1)

Figure 1.10 The pathway for JA biosynthesis

Chloroplast-localized reactions

It is generally assumed that free linolenic acid (18:3), released from plastid membrane lipids by a lipase, serves as the LOX substrate for the start of JA synthesis. However, there also exists the possibility that a membrane-associated LOX acts on esterified 18:3 prior to release from the membrane. Similarly, AOS and AOC could convert lipid-bound 13-HPOT to 12-OPDA before cleavage from the membrane. Support for this hypothesis came from the finding that OPDA and dinor OPDA (from 16:3) are found esterified to galactolipids, primarily monogalactosyl diacylglycerides (Stelmach et al., 2001; Hisamatsu et al., 2003). Evidence for LOX acting on free fatty acids comes from the observation that wounding causes an increase in free fatty acids (including 18:3) that correlates with the timing of JA accumulation (Conconi et al., 1996). The chloroplast-localized DAD1 protein of *Arabidopsis* is the only lipase known to be involved in JA biosynthesis. However, the *dad1* (*delayed anther dehiscence1*) mutant is only affected in floral JA levels suggesting that other lipases must be involved in JA synthesis in other tissues (Ishiguro et al., 2001). *In vitro* analysis of DAD1 activity demonstrated that the enzyme hydrolyzes fatty acids from the sn-1 position of galactolipids, triacylglycerol, and phospholipids. Finding the precise location of DAD1 within the chloroplast would help to understand its physiological substrate(s). The lipase responsible for the bulk of wound-induced JA biosynthesis in leaves has not yet been identified.

The three plastidic enzymes (LOX, AOS, and AOC) in JA synthesis have been studied extensively in a number of plants. Genomic sequencing and expressed sequence tag (EST) projects have shown that plants typically contain multiple LOXs. For example,

there are six genes in *Arabidopsis* and at least eight in soybean with certain isoforms involved in JA biosynthesis (Feussner and Wasternack, 2002). There is one *13-AOS* gene in *Arabidopsis*, whereas other plants contain multiple copies (Maucher et al., 2000; Howe and Schilmiller, 2002). Both 13-LOXs and 13-AOSs, which are involved in JA biosynthesis, contain chloroplast targeting sequences and are localized to the plastid (Vick and Zimmerman, 1987; Bell et al., 1995; Froehlich et al., 2001). Overexpression of *AOS* in different plants gave varying results with respect to basal JA levels. In potato, overexpression of *AOS* caused a 6 to 12-fold increase whereas in *Arabidopsis* and tobacco, no changes in basal JA levels were observed (Harms et al., 1995; Laudert et al., 2000; Park et al., 2002). Upon wounding, however, higher levels of JA accumulated in *AOS* overexpressing plants. This suggested that substrate is limiting for AOS in unwounded plants and only after wounding does AOS activity become limiting.

AOC activity was first characterized in corn (Hamberg and Fahlstadius, 1990), and the first *AOC* gene was cloned from tomato (Ziegler et al., 2000). Immunocytochemical studies showed AOC protein is located in the plastid (Ziegler et al., 2000). Although allene oxide products of 13-AOS can spontaneously cyclize to 12-OPDA, AOC is required for synthesis of the correct stereoisomer of 12-OPDA. There is a single *AOC* gene in tomato and barley, whereas four *AOC* genes are present in *Arabidopsis* (Ziegler et al., 2000; Stenzel et al., 2003; Maucher et al., 2004). When *AOC* was overexpressed in tomato, basal JA levels were not elevated in leaves, but higher levels of JAs (including OPDA, JA, and MeJA) accumulated in various floral organs. This indicated different points of regulation for the JA pathway in different tissues (Miersch et al., 2004).

Peroxisome-localized reactions

The final steps of the JA biosynthetic pathway take place in the peroxisome where 12-OPDA is converted to JA. For this to occur, 12-OPDA has to move from the plastid to the peroxisome. It is not known how 12-OPDA is transported from the plastid, however a recent study has implicated the PXA1 peroxisomal ABC-transporter in movement of 12-OPDA into the peroxisome (Theodoulou et al., 2005). JA biosynthesis after wounding in a *pxa1* null mutant was reduced approximately 50% compared to wild type. Therefore, other routes of 12-OPDA entry into the peroxisome seem likely.

Once in the peroxisome, OPR3 catalyzes the reduction of the cyclopentenone ring of 12-OPDA to yield OPC-8:0. Although plants contain a small family of *OPR* genes, only one isoform in *Arabidopsis* and tomato is capable of producing the specific isomer of 12-OPDA that is a precursor for JA (Schaller et al., 2000; Strassner et al., 2002). *In vitro* enzyme assays indicate that OPR3 is active with free 12-OPDA. This result, together with the occurrence of detectable free OPC-8:0 *in planta*, suggests that OPC-8:0 is converted to OPC-8:0-CoA for entry into the β-oxidation cycle. A recent *in vitro* study demonstrated that certain peroxisome-localized acyl activating enzymes similar to 4-coumarate:CoA ligase (4CL) can synthesize CoA esters of 12-OPDA and OPC-8:0 (Schneider et al., 2005). However, there is currently no genetic evidence that these 4CL-like enzymes function in JA biosynthesis.

Vick and Zimmerman's studies on the metabolism of [^{18}O]-12-OPDA suggested a role for β-oxidation in conversion of 12-OPDA to JA (Vick and Zimmerman, 1984). Only recently, however, have specific β-oxidation enzymes been implicated in this stage

of the JA biosynthetic pathway. β-oxidation in plants takes place in the peroxisome and involves the action of three enzymes: acyl-CoA oxidase (ACX), the multi-functional protein (MFP, possessing 2-trans-enoyl-CoA hydratase and L-3-hydroxyacyl-CoA dehydrogenase activities), and 3-keto-acyl-CoA thiolase (KAT) (Graham and Eastmond, 2002). ACXs are encoded by a small gene family in plants, with six genes in *Arabidopsis* and at least five in tomato (Adham et al., 2005; Li et al., 2005). In *Arabidopsis*, two *MFP* and four *KAT* genes are present in the genome (Graham and Eastmond, 2002). Recent work in *Arabidopsis* implicates ACX1 and KAT2 in JA production. However, incomplete suppression of JA levels in mutants or transgenic antisense plants altered in *ACX1* or *KAT2* expression suggests that multiple isoforms contribute to JA synthesis (Castillo et al., 2004; Pinfield-Wells et al., 2005). The identification and characterization of tomato and *Arabidopsis* ACXs required for JA biosynthesis is the focus of chapters 4 and 5, respectively, of this dissertation.

JA synthesis in the peroxisome presumably requires a thioesterase to cleave JA-CoA. Characterization of peroxisomal acyl-CoA thioesterases to date has shown activity with medium- to long-chain acyl-CoAs or with β-hydroxyisobutryl-CoAs that function in valine catabolism (Zolman et al., 2001; Tilton et al., 2004). It is not yet known if these enzymes function in cleavage of JA-CoA as well.

Metabolism of JA

JA is subject to a number of metabolic transformations that may play a role in fine tuning the activity of the hormone. The routes of modification include: methylation at C_1 to give MeJA; hydroxylation at C_{11} or C_{12} to form 11-hydroxyjasmonic acid (11-OHJA)

or 12-hydroxyjasmonic acid (12-OHJA, tuberonic acid); sulfation of 11-OHJA or 12-OHJA to produce hydroxy-JA sulfate; reduction of the keto group at C_6 to form cucurbic acid; O-glycosylation of the above mentioned hydroxylated jasmonates; conjugation of the carboxyl group of JA to amino acids; and degradation of the carboxyl group to form cis-jasmone (Schaller et al., 2004).

Several enzymes catalyzing JA modifications have been identified. JA-methyl transferase (JMT) identified from *Arabidopsis* catalyzes the methylation of JA. When JMT is overexpressed, MeJA levels are increased resulting in constitutive activation of defense-related gene expression (Seo et al., 2001). The opposite reaction, cleavage of the methyl group from MeJA, is catalyzed by a JA methyl esterase (JME) (Swiatek et al., 2004). A gene encoding a putative JME was recently cloned from tomato (Stuhlfelder et al., 2004). The *Arabidopsis* JAR1 protein is responsible for conjugation of JA with amino acids, most likely with isoleucine *in vivo* (Staswick and Tiryaki, 2004). A *jar1* mutant was isolated based on the reduction in JA-mediated root growth inhibition (Staswick et al., 1992). The reduced sensitivity of *jar1* mutants to JA, and the increase in susceptibility of *jar1* to *Pythium irregulare*, suggests that JA-Ile is an active signal for some jasmonate-mediated responses (Staswick et al., 1998). A sulfotransferase with activity for sulfation of 11-OHJA and 12-OHJA was identified from *Arabidopsis*, and it was suggested that this enzyme may serve to inactivate JA (Gidda et al., 2003).

III. Physiological roles of jasmonates

Work by Green and Ryan demonstrated that wounding induces local and systemic expression of proteinase inhibitor (PI) proteins that inhibit the activity of digestive

enzymes in the gut of herbivores (Green and Ryan, 1972). The Ryan group went on to show that application of JAs causes the induction of *PI* gene expression (Farmer and Ryan, 1990; Farmer et al., 1992). These properties of PIs led to their extensive use as a marker to study systemic wound signaling in tomato (Howe, 2004). Grafting experiments with JA biosynthetic and JA perception mutants of tomato indicated that JA acts an essential component of the long distance signal for activation of systemic responses (Li et al., 2002). In some *Solanaceous* species, small peptides (named systemin in tomato) are involved in the signaling pathway for the wound response. The current model in tomato suggests that systemin works locally at the wound site to activate the synthesis of JA, which acts as a phloem-mobile signal to promote systemic responses (Figure 1.11) (Schilmiller and Howe, 2005). Other factors such as cell wall-derived oligosaccharides and fatty acid-amino acid conjugates (from insect regurgitant) have also been found to induce the synthesis of JA in plants.

Role of JAs in defense

JA-mediated plant defense responses have been classified as being direct or indirect. Direct defenses include induced phytochemicals and proteins that directly inhibit feeding, growth, or reproduction of herbivores (Halitschke and Baldwin, 2004). Indirect JA-mediated defenses include volatiles that attract predators that parasitize insect herbivores. A major portion of our knowledge concerning the role of JAs in defense has come from work with mutants affected in JA synthesis and perception. Tomato and *Arabidopsis* mutants affected in JA signaling show increased susceptibility to various herbivores (Howe et al., 1996; McConn et al., 1997; Li et al., 2002; Li et al., 2003).

Figure 1.11 Production of JA in vascular bundles of tomato leaves. The location of JA biosynthetic enzymes in companion cells (cc) and sieve elements (se) along with localization of prosystemin (ps) to phloem parenchyma (pp) cells supports the hypothesis that JA is produced and transported in the phloem.

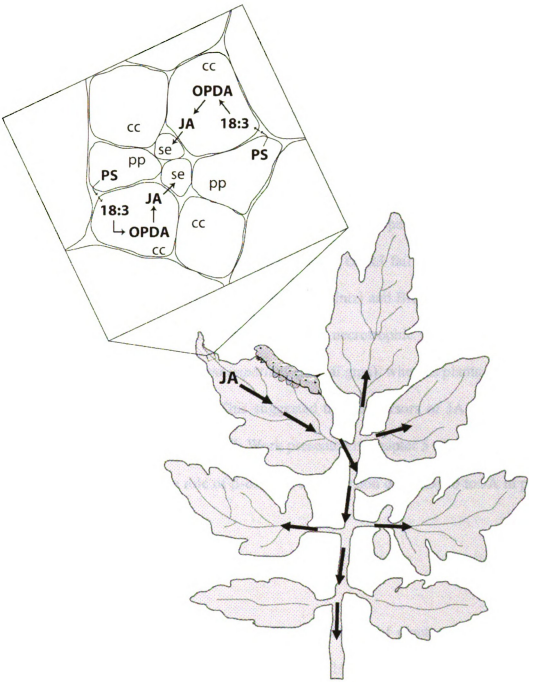

Figure 1.11 Production of JA in vascular bundles of tomato leaves

Similarly, JA-deficient tobacco plants with suppressed 13-LOX expression exhibit reduced resistance to tobacco hornworm feeding (Halitschke and Baldwin, 2003). Ecological studies with the suppressed 13-LOX tobacco lines showed the plants became susceptible to two herbivores that normally do not feed on tobacco. This suggests that JA is required for basal resistance as well as induced resistance (Kessler et al., 2004). This phenomenon was also found for JA mutants exhibiting susceptibility to certain pathogens (Vijayan et al., 1998; Thomma et al., 2001). While it is clear JAs are required for defense against herbivores and pathogens, exactly which JAs promote specific defense responses is not fully understood. *Arabidopsis* plants defective in *OPR3* fail to synthesize JA but accumulate the cyclopentenone precursor 12-OPDA (Stintzi and Browse, 2000; Stintzi et al., 2001). The *opr3* mutant retains resistance to the necrotrophic pathogen *Alternaria brassicicola* and to the insect *Bradysia impatiens* (fungal gnat), whereas plants lacking all JAs were susceptible to these pests. This suggested that precursors of JA (such as 12-OPDA) have signaling properties as well. Work presented in chapter 5 of this dissertation is focused on understanding the role of β-oxidative conversion of OPC-8:0 to JA in plant defense.

Role of JAs in plant development

Early interest in JAs was bolstered by observations of plant growth-regulating properties of JAs (Ueda and Kato, 1980; Dathe et al., 1981). To date, several aspects of plant growth and development have been associated with JAs including root growth inhibition, senescence, floral development, tendril coiling, and tuberization (Wasternack and Hause, 2002). One of the best characterized processes in which JAs are involved is

floral development. An *Arabidopsis* mutant (*fad3/fad7/fad8*) deficient in trienoic fatty acid synthesis was found to be male sterile due to pollen inviability and delayed dehiscence of pollen (McConn and Browse, 1996). This defect was rescued by application of JA, indicating that a defect in JA biosynthesis was the cause of male sterility. Subsequent work with the *Arabidopsis opr3* mutant showed that the requirement for JA in male fertility cannot be compensated with 12-OPDA (Sanders et al., 2000; Stintzi and Browse, 2000).

This dissertation is focused on the characterization of two enzymes involved in oxylipin biosynthesis. Chapter 2 presents the characterization of a tomato CYP74 cytochrome P450 with 9-AOS activity. *LeAOS3* represents the first cloned gene that encodes an AOS that is active in the 9-LOX pathway. Chapter 3 describes the regulation of *LeAOS3* in roots and hypocotyls of germinating seedlings. Also included in this chapter are the results from an expression profiling study to identify genes that are co-regulated with *LeAOS3* in roots. Chapters 4 and 5 describe the characterization of tomato and *Arabidopsis* mutants affected in ACX. Chapter 4 is focused on the tomato ACX1A, whereas chapter 5 presents the isolation and characterization of *Arabidopsis* ACX mutants impaired in JA biosynthesis.

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Chapter 2

Identification and Biochemical Characterization of a New Tomato Allene Oxide Synthase

The work presented in this chapter has been published:

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Abstract

Allene oxide synthase (AOS) is a cytochrome P-450 (CYP74A) that catalyzes the first step in the conversion of 13-hydroperoxy linolenic acid to jasmonic acid and related signaling molecules in plants. Here, we report the molecular cloning and characterization of a novel AOS-encoding cDNA (*LeAOS3*) from *Lycopersicon esculentum* whose predicted amino acid sequence classifies it as a member of the CYP74C subfamily of enzymes that was hitherto not known to include AOSs. Recombinant LeAOS3 expressed in *Escherichia coli* showed spectral characteristics of a P-450. The enzyme transformed 9- and 13-hydroperoxides of linoleic and linolenic acid to α-ketol, γ-ketol, and cyclopentenone compounds that arise from spontaneous hydrolysis of unstable allene oxides, indicating that the enzyme is an AOS. Kinetic assays demonstrated that LeAOS3 was ~10-fold more active against 9-hydroperoxides than the corresponding 13-isomers. These findings suggest that LeAOS3 plays a role in the metabolism of 9-lipoxygenase-derived hydroperoxides.

Introduction

Oxylipins comprise a group of biologically active compounds that are produced by oxidative metabolism of polyunsaturated fatty acids. Members of the eicosanoid family of lipid mediators have been studied extensively with respect to their biosynthesis from arachidonic acid and their function in diverse physiological processes in animal cells (Smith et al., 2000). In plants, which lack arachidonic acid, oxygenated derivatives of C18 fatty acids participate in the regulation of many defense-related and developmental processes. The biosynthesis of most phytooxylipins is initiated by lipoxygenase (LOX), which adds molecular oxygen to either the C-9 or C-13 position of linolenic or linoleic acid (Feussner and Wasternack, 2002). The resulting hydroperoxides are further metabolized by several enzymes including three closely related members of the CYP74 family of cytochromes P-450: allene oxide synthase (AOS), hydroperoxide lyase (HPL), and divinyl ether synthase (DES). Indeed, much of the structural and functional diversity in oxylipin metabolism in plants can be accounted for by the activity of CYP74s that metabolize 9- and 13-hydroperoxides to a wide range of products (Howe and Schilmiller, 2002). In contrast to typical P-450 monooxygenases, CYP74 P-450s do not require O_2 and a NADPH-dependent P-450 reductase for activity. Rather, they use a hydroperoxide group both as the oxygen donor and as a source of reducing equivalents (Song and Brash, 1991; Song et al., 1993). This unique catalytic feature is shared by thromboxane synthase and prostacyclin synthase, two P-450 enzymes involved in the synthesis of eicosanoids (Hecker and Ullrich, 1989). A greater understanding of the biochemical and physiological function of this atypical class of P-450 enzymes promises

to provide new insight into the evolution of fatty acid-based signaling pathways in diverse biological systems.

Interest in plant oxylipins has focused mainly on the AOS branch of the 13-LOX pathway that gives rise to the jasmonate family of compounds (collectively referred to as JAs) including JA, methyl-JA (MeJA), and their metabolic precursor 12-oxo-10,15-phytodienoic acid (12-OPDA) (Fig. 2.1). AOS catalyzes the transformation of 13-hydroperoxy linolenic acid (13-HPOT) to a short-lived allene oxide intermediate, 12,13-epoxyoctadecatrienoic acid (12,13-EOT) (Brash et al., 1988; Hamberg, 1989). In the biosynthetic route to JA, allene oxide cyclase (AOC) converts 12,13-EOT to 12-OPDA (Hamberg, 1988). Reduction of 12-OPDA by OPDA reductase (OPR) and three cycles of β-oxidation yields JA (Vick and Zimmerman, 1983, 1984). During in vitro reactions carried out in the absence of AOC, 12,13-EOT spontaneously hydrolyzes to α- and γ-ketols, and can also undergo non-enzymatic cyclization to produce racemic 12-OPDA (Brash et al., 1988; Song and Brash, 1991). The physiological significance of α- and γ-ketols, and the extent to which they are produced in vivo, is unclear. Genes encoding AOSs that metabolize 13-HPOT have been cloned from several plant species. Based on comparisons of predicted amino acid sequence, these proteins are classified within the CYP74A subfamily of P-450s (Feussner and Wasternack, 2002; Howe and Schilmiller, 2002). Several AOSs have been shown to have a strong (~36:1) preference for the 13-hydroperoxide relative to the corresponding 9-isomer (Feng and Zimmerman, 1979; Howe et al., 2000). One exception to this is barley AOS, which despite its classification as a CYP74A and proposed role in JA biosynthesis can metabolize both 9- and 13-isomers in vitro (Maucher et al., 2000).

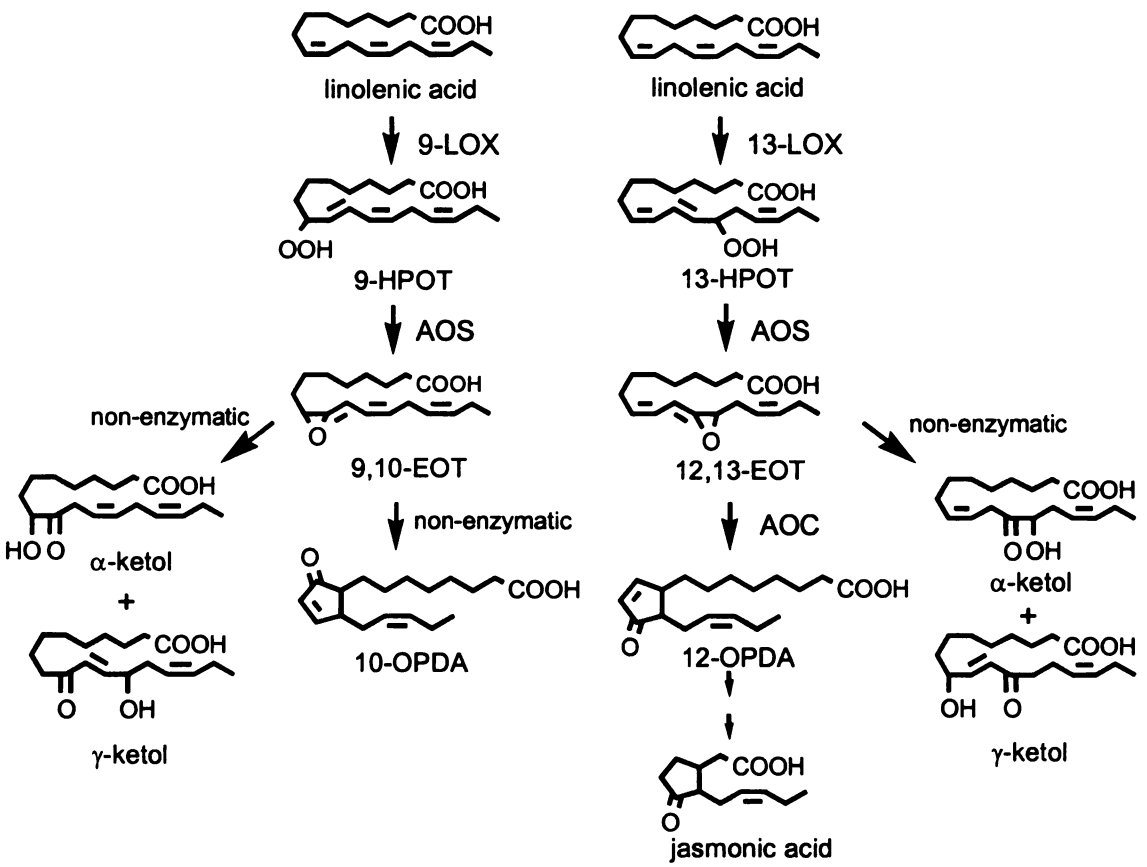

Figure 2.1 Oxylipins derived from allene oxide synthase. The pathways shown are for the metabolism of linolenic acid by the AOS branch of the 9-LOX and 13-LOX pathways.

Plants also utilize a second type of AOS for the metabolism of 9-hydroperoxides in non-photosynthetic tissues (Fig. 2.1). An AOS activity responsible for production of α- and γ-ketol fatty acids from 9-hydroperoxy linoleic acid (9-HPOD) was first demonstrated in extracts from corn seed (Gardner, 1970; Gardner et al., 1975). Subsequent studies showed that these compounds are formed by non-enzymatic hydrolysis of an unstable allene oxide (9,10-epoxyoctadecanoic acid; 9,10-EOD) that is generated by the action of AOS on 9-hydroperoxides (Hamberg and Hughes, 1988; Hamberg, 2000). More recently, the production of ketols by the sequential action of 9-LOX and AOS was demonstrated in cell-free extracts from tomato root (Caldelari and Farmer, 1998), tulip bulb (Grechkin et al., 2000), and potato stolon (Hamberg, 2000). The latter study (Hamberg, 2000) also showed that metabolism of linoleic and linolenic acids via the 9-LOX/AOS pathway gives rise to 10-oxo-11-phytoenoic acid (10-OPEA) and 10-oxo-11,15-phytodienoic acid (10-OPDA), respectively, novel cyclopentenones that are structural isomers of 12-OPDA (Fig. 2.1).

The presence of 9-AOS in plants raises the question of the physiological function of this branch of oxylipin metabolism and its relationship to AOSs that act in the 13-LOX pathway for JA biosynthesis. Here we describe the identification and functional characterization of a cDNA encoding a novel member of the CYP74 P-450 gene family in tomato. We demonstrate that the recombinant protein (designated LeAOS3) is an AOS by virtue of its ability to convert hydroperoxy fatty acids to ketol and cyclopentenone oxylipins. The phylogenetic classification of LeAOS3 within the CYP74 family and the substrate preference of the enzyme lead us to propose a role for this P-450 in the production of 9-hydroperoxide-derived oxylipins.

Materials and Methods

Expression and purification of recombinant LeAOS3

A construct for expression of LeAOS3 in *E. coli* was made using a PCR-based method with a full-length EST (cLEI16I2) used as template. Forward and reverse primers were designed to contain *Nde*I and *Xho*I restriction sites, respectively to facilitate cloning into the pET23b (Novagen, Madison, WI) expression vector. The sequence of the forward primer was 5'-GGA-ATT-CCA-TAT-GGC-TAA-TAC-CAA-AGA-3' and that of the reverse primer, 5'-CCG-CTC-GAG-TGA-TGT-TGC-TTT-AG-3'. Following PCR amplification, the 1.45-kb product was cut with *Nde*I and *Xho*I and subsequently ligated into pET23b digested with the same enzymes. The resulting construct, which adds eight amino acids (LEHHHHHH) to the C-terminus of LeAOS3, was transformed into the *E. coli* host BL21(DE3).

His-tagged recombinant LeAOS3 was expressed in BL21(DE3) host cells as follows. An overnight culture (2 ml) of bacteria was inoculated into 200 ml of Terrific Broth (TB) medium supplemented with 100 μg/ml ampicillin. Bacteria were grown at 37°C in a shaker at 250 rpm to an OD_{600} of 0.5. Cultures were cooled to 25°C, and isopropyl-thio-β-D-galactopyranoside was added to a final concentration of 0.1 mM. Induced cultures were incubated for 24 h at 25°C with gentle shaking (120 rpm). Cells were collected by centrifugation and stored at -20°C until further use. Purification of recombinant LeAOS3 was performed as described previously (Itoh and Howe, 2001) with minor modification. Following centrifugation (100,000 x *g* for 60 min) of the cleared *E. coli* lysate, the recombinant protein was solubilized from the particulate

fraction using 1.5% Triton X-100R. Solubilized protein was further purified using TALON metal affinity column (cobalt-based IMAC, Clontech) and subsequent elution with imidazole. Imidazole was removed from the protein sample using a 2.5-ml spin column prepared with Sephadex G-25 (Amersham Pharmacia, Piscataway, NJ) equilibrated with 50 mM sodium phosphate (pH 7.0), 5% glycerol, and 0.02% Triton X-100R. Protein measurements were performed using a BCA assay (Pierce, Rockford, IL) and bovine serum albumin as a standard. The relative purity of recombinant LeAOS3 was estimated by SDS-polyacrylamide gel electrophoresis (10% polyacrylamide) and staining of gels with Coomassie Brilliant Blue R-250.

Product analysis

Affinity purified LeAOS3 (30 μg protein) was incubated with 250 μg fatty acid hydroperoxide substrate (Cayman Chemical, Ann Arbor, MI) in 1 ml 0.1 M sodium phosphate buffer (pH 7.0) containing 5% (v/v) glycerol. Reactions proceeded for 5 min at 25°C and then were stopped by acidification to pH 4.0 with 1 M citrate. Products were extracted twice with chloroform, dried under N_2 gas, and resuspended in 0.25 ml of methanol. Compounds were methylated by treatment with ethereal diazomethane at 25°C for 10 s and dried under N_2 gas. Samples subjected to silylation were treated with 30 μl of the following mixture: pyridine/hexamethyldisilazane/trimethylchlorosilane at a ratio of 2/1/2 (v/v/v) at 25°C for 20 min (Gemzell-Danielsson and Hamberg, 1994). Following removal of excess reagent under vacuum, the remaining residue was dissolved in hexane and analyzed by gas chromatography-mass spectrometry (GC-MS) as previously described (Howe et al., 2000), with the following modifications. The temperature

program was initiated at 50°C, ramped to 320°C at 10°C min^{-1}, and maintained at 320°C for 2 min. Stereochemical analysis of products was not attempted. However, it was noted that incubation of LeAOS3 with hydroperoxides yielded two cyclopentenone products, presumably diastereomers, which were separable by GC and gave identical mass spectra. The peak area of these two compounds was added together to compute the total cyclopentenone composition. The mass spectrum of 12-OPDA produced from LeAOS3-catalyzed metabolism of 13-HPOT was identical to that of an authentic 12-OPDA standard (Cayman Chemical).

Biochemical analysis of LeAOS3

The hydroperoxide-metabolizing activity of recombinant LeAOS3 was measured spectrophotometrically by monitoring the rate of decrease in absorbance at 234 nm resulting from disruption of the conjugated diene bond of the substrate (Zimmerman and Vick, 1970). Kinetic assays were performed at 30°C in 1 ml of 100 mM sodium phosphate (pH 7.0) containing 30 ng or 60 ng of purified LeAOS3 and varying concentrations of hydroperoxide substrate (Cayman Chemical). Activity slopes obtained during the first 0.5 min of the reaction were used for calculation of kinetic parameters. Absorbance spectra were obtained using purified recombinant protein in 1 ml of 100 mM sodium phosphate buffer (pH 7.0). Carbon monoxide treatments were performed by bubbling CO gas through the sample for 1 min. The protein was reduced by the addition of a few grains of sodium dithionite.

RNA blot analysis

RNA blot analysis was performed as previously described (Howe et al., 2000). Total RNA was extracted from various tissues of soil-grown plants. The full-length cDNA clone (cLEI16I12) was used to generate a probe for *LeAOS3*. RNA quality and equal loading was assessed by staining a duplicate gel with ethidium bromide (EtBr).

Results

Identification of a new member of the CYP74 family of cytochrome P450s

A search of the tomato EST database (www.tigr.org/tdb/lgi/) was conducted to identify potential new members of the CYP74 gene family in tomato. A tentative consensus sequence (Quackenbush et al., 2000), constructed from multiple overlapping EST clones, was identified that was similar to but clearly distinct from previously characterized CYP74 sequences in tomato and other plant species. DNA sequence analysis of one EST clone (cLEI16I12) revealed a 1772-bp cDNA insert that contained an open reading frame predicted to encode a 491-amino acid protein, having a calculated molecular weight of 55,513. In keeping with the previous nomenclature of tomato CYP74s (Howe and Schilmiller, 2002) and the biochemical characteristics of the protein (see below), we henceforth refer to the cDNA as *LeAOS3* (*L. esculentum AOS3*). The deduced amino acid sequence of LeAOS3 showed several features that distinguish CYP74s from P-450 monooxygenases (Fig. 2.2). For example, the I-helix region of P-450 monooxygenases contains an invariant threonine residue that serves an important role in the binding and activation of oxygen (Chapple, 1998; Paquette et al., 2000). All reported

Figure 2.2 Comparison of cDNA-deduced protein sequences of CYP74 P450s in tomato. Sequences were aligned using the ClustalW1.8 program available at *SearchLauncher.bcm.tmc.edu.* Sequences shown are LeAOS1 (AJ271093; CYP74A), LeAOS2 (AF230371; CYP74A), LeAOS3 (AF454634; CYP74C), LeHPL (AF230372; CYP74B), and LeDES (AF317515; CYP74D). Black and shaded boxes in the alignment indicate positions that contain an identical and conserved amino acid, respectively. The ^ symbol denotes the position of the conserved threonine found in the I-helix of P-450 monooxygenases. The CYP74 consensus sequence surrounding the cysteinyl heme ligand (*) is underlined.

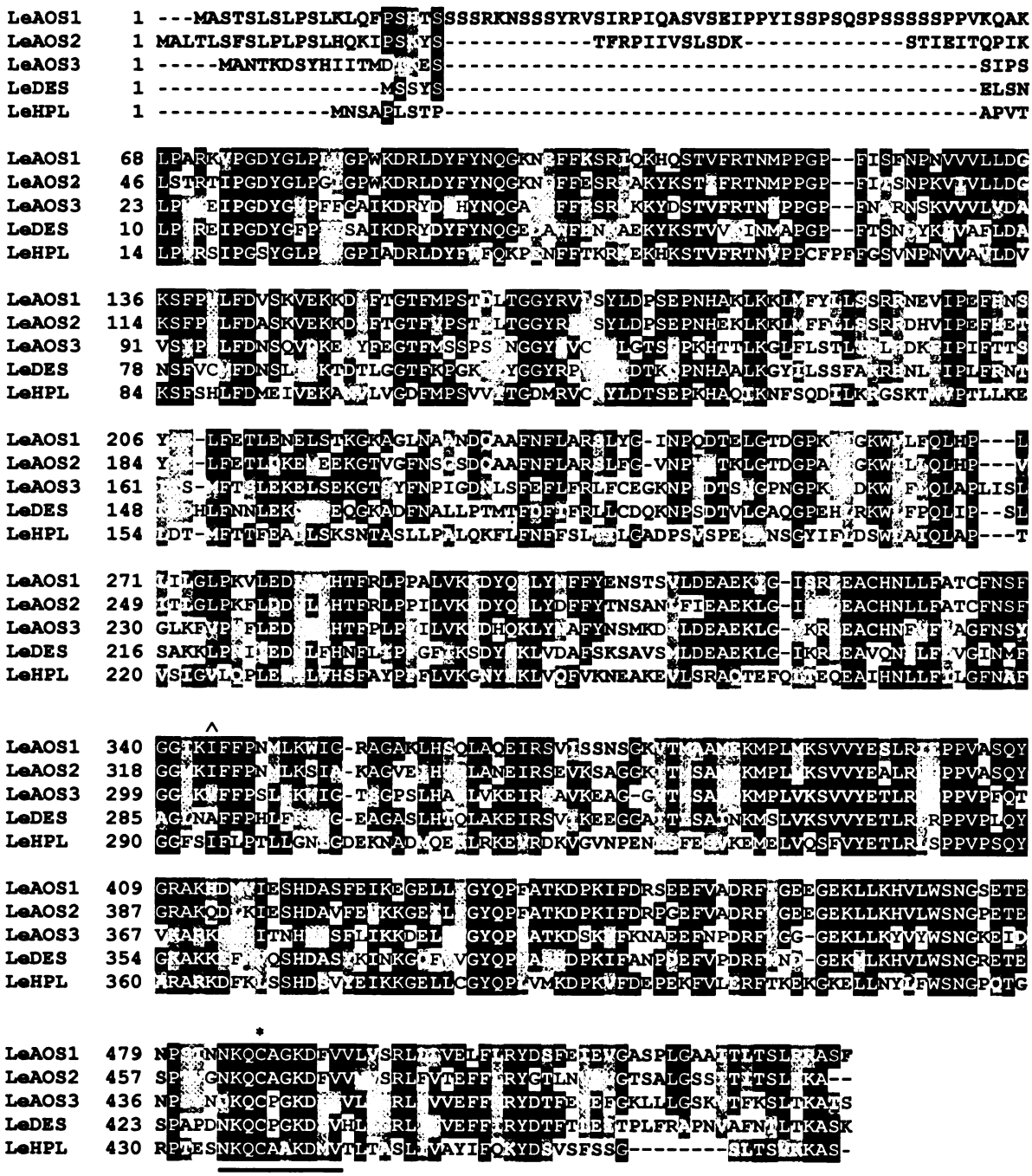

Figure 2.2 Comparison of cDNA-deduced protein sequences of CYP74 P450s in tomato.

CYP74s, including LeAOS3, contain a small hydrophobic residue (I/A/V) at this position. The CYP74 consensus sequence for residues surrounding the cysteinyl heme ligand near the C terminus is NKQC(A/P)(G/A)K(D/N)XV. With the exception of a V → I substitution in the last position, this sequence is conserved in LeAOS3. Analysis of the deduced amino acid sequence of LeAOS3 using the ChloroP program (www.cbs.dtu.dk/services/ChloroP/) suggested that the protein does not contain a predicted N-terminal targeting sequence that directs many, but not all (Maucher et al., 2000; Froehlich et al., 2001), CYP74 P-450s to the chloroplast.

Phylogenetic analysis was used to gain insight into the functional relationship between LeAOS3 and extant members of the CYP74 family, which is comprised of four subfamilies (CYP74A through CYP74D). An unrooted neighbor-joining phylogeny indicated that CYP74 members fall into distinct groups that reflect, to a large extent, the known enzymatic identity and substrate specificity of individual members (Figure 2.3). This phylogeny was in perfect agreement with that obtained using a maximum parsimony algorithm (not shown). The CYP74A subfamily, which forms a monophyletic cluster with monocots positioned basally to the dicots, consists mainly of AOSs that have specificity for 13-hydroperoxides (*i.e.* 13-AOSs). The CYP74B subfamily also forms a well-defined group comprised of HPLs that have specificity for 13-hydroperoxides (*i.e.* 13-HPLs). CYP74C P450s from cucumber (CsHPL) and melon (CmHPL) are HPLs that have a preference, but not absolute specificity, for 9-hydroperoxides (Matsui et al., 2000; Tijet et al., 2001). Members of the CYP74D subfamily are DESs that have high specificity for 9-hydroperoxides (Itoh and Howe, 2001; Stumpe et al., 2001). LeAOS3 was most similar to the mixed specificity HPLs (CYP74Cs) from melon (58% identity)

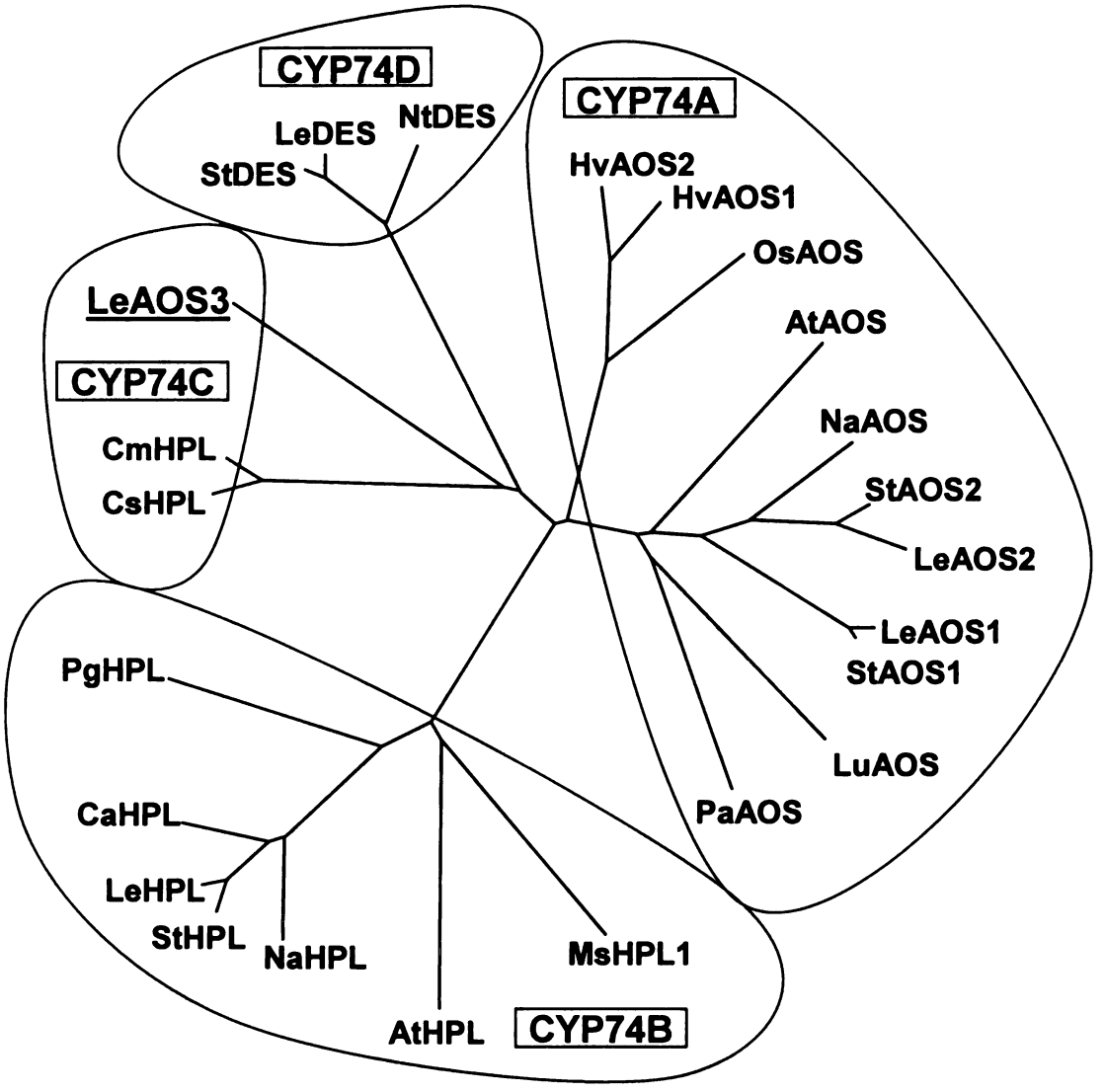

Figure 2.3 Phylogenetic analysis of plant CYP74 sequences. An unrooted neighbor-joining phylogeny was created in PAUP4.0 from the deduced amino acid sequences of representative CYP74 P-450s. Enclosed groups indicate the four different CYP74 subfamilies (CYP74A through CYP74D).

and cucumber (57% identity). In accordance with cytochromes P-450 nomenclature, LeAOS3 was classified as a CYP74C and was assigned the name CYP74C3.

Biochemical properties of LeAOS3

The full-length *LeAOS3* cDNA was subcloned into an expression vector (pET23b) that adds a His_6 tag to the C-terminus of the protein. Following introduction of this construct into *E. coli*, recombinant LeAOS3 was expressed and purified using metal affinity chromatography. The majority of LeAOS3 was found in inclusion bodies in the 3,000 x *g* pellet after centrifugation of sonicated cells. Approximately 80% of enzyme activity found in the 3,000 x *g* supernatant was subsequently recovered in the 100,000 x *g* pellet, indicating that the active protein is associated with bacterial membranes. Solubilization of membranes with Triton X-100R followed by cobalt-affinity chromatography allowed purification of LeAOS3 to ~95% homogeneity (Fig. 2.4). The apparent molecular weight of His_6-tagged LeAOS3, as determined by SDS-polyacrylamide gel electrophoresis, was in good agreement with the calculated molecular weight of 56,578. The typical yield of affinity-purified LeAOS3 was 0.5 mg/liter of *E. coli* culture.

A spectrophotometric assay (Zimmerman and Vick, 1970) was used to determine whether purified LeAOS3 could metabolize fatty acid hydroperoxides (Fig. 2.5A). The results showed that LeAOS3 was highly active against 9-HPOD. The enzyme also metabolized 13-hydroperoxy linoleic acid (13-HPOD), albeit at a rate ~12-fold lower than that observed for 9-HPOD. A similar preference for 9-hydroperoxy linolenic acid (9-HPOT) compared with 13-HPOT was also observed (not shown; see below). These

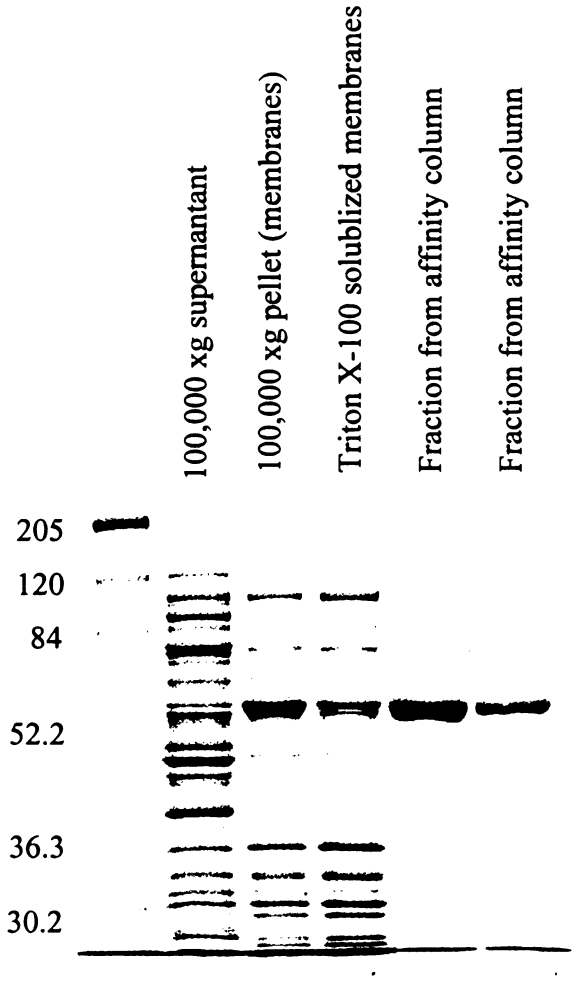

Figure 2.4 Affinity purification of LeAOS3 expressed in *E. coli*. Protein fractions obtained during the purification of His-tagged LeAOS3 were analyzed by SDS-polyacrylamide gel electrophoresis. A coomassie stained gel is shown with molecular weight marker sizes indicated at left. Crude cell extract was centrifuged at 100,000 x g to give a soluble fraction and membrane enriched fraction. Membranes were solublized using Triton X-100R and spun again at 100,000 x g and the supernatant containing solubilized membrane protein was loaded onto a Co-affinity column. After washing, fraction were eluted from the column with imidazole.

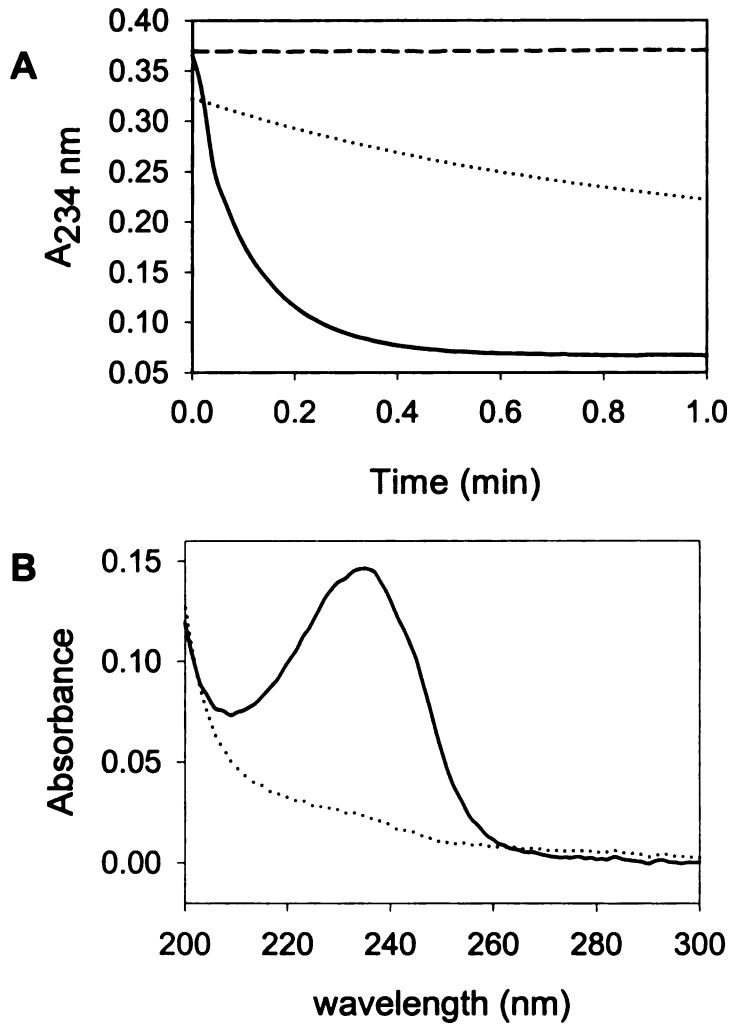

Figure 2.5 Metabolism of fatty acid hydroperoxides by LeAOS3. (A) utilization of 9-HPOD (solid line) and 13-HPOD (dotted line) by LeAOS3 in a spectrophotometric assay that monitors the loss of absorbance at 234 nm of the substrate. 9-HPOD was not metabolized by crude extract from E. coli harboring the empty expression vector (dashed line). (B) UV spectra were taken before (solid line) and 5 minutes after (dotted line) addition of LeAOS3 to a reaction using 9-HPOD.

results indicate that LeAOS3 is active against both 13- and 9-hydroperoxy fatty acids, but with a marked preference for the latter substrates.

The UV spectra of the reaction product generated from 9-HPOD (Fig. 2.5B) and other hydroperoxides (data not shown) were featureless, indicating that the substrate (λmax = 234 nm) was largely consumed during the reaction, and that divinyl ethers (λmax = 250–253 nm) were not among the major products. LeAOS3 also showed no detectable HPL activity using an NADH-coupled assay (Vick, 1991) that readily detected products formed by the action of recombinant tomato HPL on 13-HPOT (data not shown and (Howe et al., 2000)). These results suggested that LeAOS3 possess neither DES nor HPL activity. To determine the enzymatic identity of LeAOS3, products generated from various substrates were converted to the corresponding methyl ester/TMS derivatives and subjected to GC-MS analysis (Fig. 2.6 and Table 2.1). The major product (77.2%) obtained from reaction with 9-HPOD gave a mass spectrum that was identical to the reported spectrum (Grechkin et al., 2000) for the α-ketol, 9-hydroxy-10-oxo-12-octadecenoic acid (Fig. 2.6A). The second most abundant (8.7%) product gave a mass spectrum identical to that reported (Hamberg, 2000) for the cyclopentenone 10-OPEA (Fig. 2.6B). A third product (Fig. 2.6C), present in minor amounts (1.9%), was identified as the γ-ketol (10-oxo-13-hydroxy-11-octadecenoic acid) by comparison to published spectra of this compound (Grechkin et al., 2000). Because each of these products is known to arise from spontaneous hydrolysis of 9,10-EOD that is generated by the action of AOS on 9-HPOD (Grechkin et al., 2000; Hamberg, 2000), we conclude that LeAOS3 is an AOS. Analysis of products derived from other hydroperoxy fatty acids gave results that were consistent with the identification of LeAOS3 as an AOS (Table 2.1). For

Figure 2.6 Mass spectra of products formed by incubation of LeAOS3 with 9-HPOD. Purified LeAOS3 was incubated with 9-HPOD at 25°C for 5 min at pH 7.0. The resulting products were analyzed as the methyl ester TMS derivatives by GC-MS. Shown are spectra for the α-ketol (9-hydroxy-10-oxo-12-octadecenoic acid) (A), the cyclopentenone 10-oxo-11-phytoenoic acid (10-OPEA) (B), and the γ-ketol (10-oxo-13-hydroxy-11-octadecenoic acid) (C).

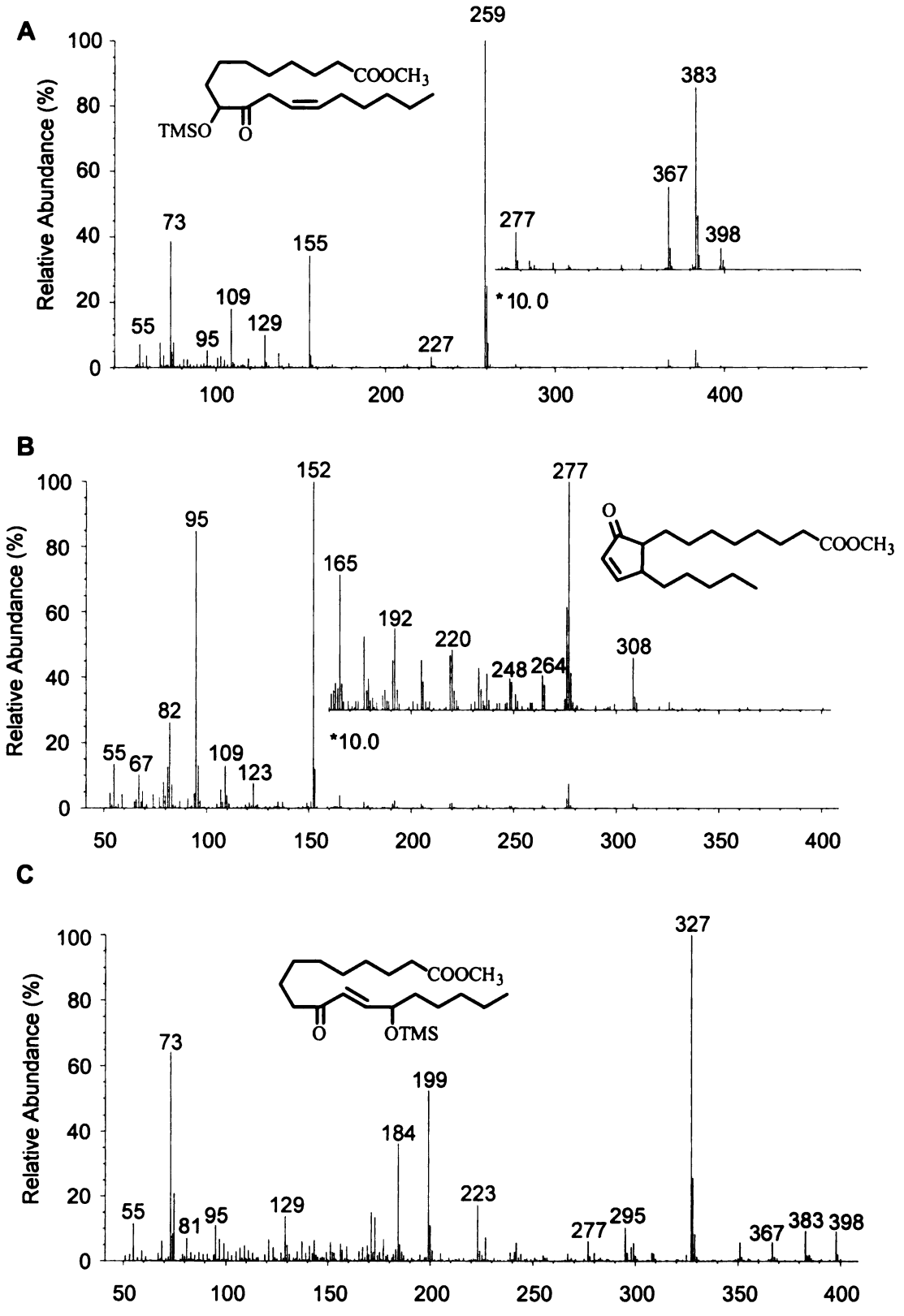

Figure 2.6 Mass spectra of products formed by incubation of LeAOS3 with 9-HPOD.

Table 2.1 Analysis of LeAOS3 reaction products by GC-MS. For analysis of products formed by AOS3, purified recombinant protein was incubated with each of the four substrates at 25 °C for 5 minutes. Following extraction and derivatization, product identity was determined by GC-MS analysis. For each identified product, its percentage of the total peak area as well as the prominent MS ions and their relative abundance are given.

Substrate	Product	Rel. abundance	m/z of ion fragments (relative abundance)
9-HPOD	α-ketol	77.2 %	398(1), 383(6), 259(100), 155(34), 129(10), 109(18), 73(37)
	γ-ketol	1.9 %	398(9), 383(10), 327(100), 295(14), 227(8), 223(17), 199(53), 184(36), 129(14), 95(12), 73(65)
	10-oxo-11-phytoenoic acid	8.7 %	308(3), 277(9), 233(2), 192(4), 152(100), 123(6), 109(14), 95(75), 82(23)
9-HPOT	α-ketol	61.1 %	396(2), 381(4), 365(2), 259(100), 227(5), 155(39), 129(11), 109(23), 95(9), 73(44)
	γ-ketol	1.8 %	381(4), 365(4), 327(100), 295(11), 223(26), 197(8), 129(8), 73(75)
	10-oxo-11,15-phytodienoic acid	3.5 %	306(1), 275(7), 238(7), 150(19), 121(18), 82(100)
13-HPOD	α-ketol	39.9 %	383(5), 367(2), 270(19), 173(100), 129(4), 103(13), 73(43)
	12-oxo-10-phytoenoic acid	8.1 %	308(7), 277(22), 238(45), 206(30), 165(15), 151(23), 109(56), 96(100), 82(52), 67(21), 55(34)
13-HPOT	α-ketol	37.4 %	381(4), 365(2), 270(21), 171(100), 129(14), 103(9), 73(42)
	12-oxo-10,15-phytodienoic acid	12.5 %	306(33), 275(32), 238(54), 206(22), 163(54), 149(36), 121(36), 107(65), 96(86), 95(100), 82(51), 67(44), 55(53)

instance, when LeAOS3 was incubated with 9-HPOT, the reaction product contained the corresponding α-ketol (9-hydroxy-10-oxo-12–15-octadecadienoic acid, 61.1%), γ-ketol (10-oxo-13-hydroxy-11–15-octadecadienoic acid, 1.8%), and the cyclopentenone 10-OPDA (3.5%). Upon incubation of LeAOS3 with 13-HPOD or 13-HPOT, the major products of catalysis were the corresponding α-ketol and cyclopentenone oxylipins. The UV-visible spectrum of purified LeAOS3 was typical of the low spin ferric state, and showed a main Soret band at 418 nm (Fig. 2.7). Reduction of the protein with sodium dithionite and treatment with CO resulted in the appearance of a spectral peak (448 nm) that is a hallmark of cytochromes P-450. Similar spectra have been observed for other CYP74 P-450s including flaxseed AOS, melon HPL, and tomato DES (Song et al., 1993; Itoh and Howe, 2001; Tijet et al., 2001). The persistence of the 420-nm peak in the LeAOS3 difference spectrum (Fig. 2.7, *inset*) was also observed in difference spectra recorded on membranes from LeAOS3-expressing *E. coli* cells (data not shown). These results suggest that the P-420 species does not result from inactivation of the enzyme by a step (*e.g.* elution with imidazole) during affinity purification. Rather, the P-420 form may reflect improperly folded protein or the weak interaction of CO with the active site of CYP74 P-450s (Song and Brash, 1991; Lau et al., 1993).

Purified LeAOS3 was used to determine the kinetic parameters of reactions conducted with 9-HPOD, 9-HPOT, 13-HPOD, and 13-HPOT (Table 2.2). The apparent K_m of all four substrates ranged between 4 μM (13-HPOT) and 21 μM (9-HPOD). K_m values for 9-hydroperoxides were 2–4-fold higher than those for the corresponding 13-hydroperoxides. LeAOS3 was most active against 9-HPOD, as determined both by the estimated turnover rate (k_{cat}) and catalytic efficiency (k_{cat}/K_m). The estimated k_{cat} value

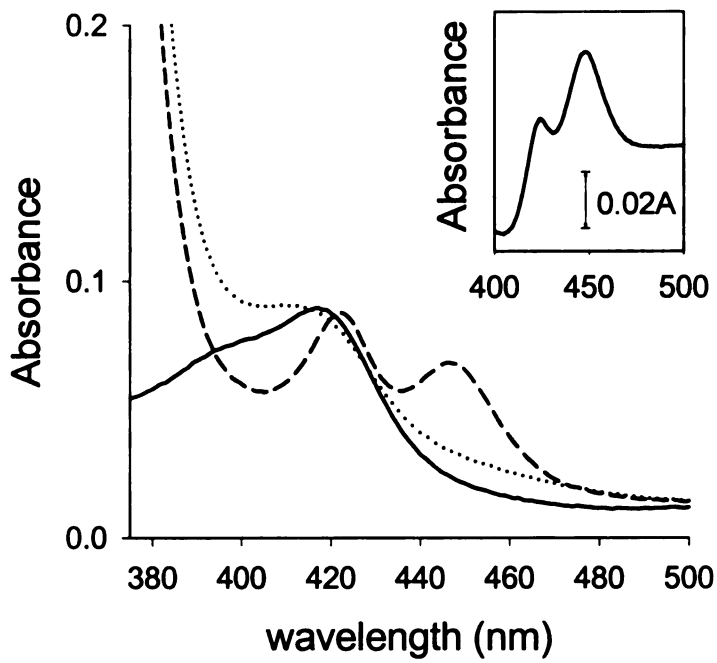

Figure 2.7 Carbon monoxide difference spectrum of recombinant LeAOS3. All spectra were recorded using 100 μg of purified LeAOS3 in 1 ml of 0.1 M sodium phosphate buffer (pH 7.0). The spectra shown are from the native protein (solid line), protein after reduction with sodium dithionite (dotted line), and reduced protein after bubbling with CO for 1 minute (dashed line). The inset shows the difference spectrum obtained by subtracting the reduced protein spectrum from the CO-treated protein spectrum.

Table 2.2 Substrate specificity of recombinant LeAOS3

Substrate	K_m (μM)	K_{cat} (s^{-1})	K_{cat}/K_m ($M^{-1}s^{-1}$)
9-HPOD	21 ± 5	820 ± 93	39×10^6
9-HPOT	16 ± 5	236 ± 27	15×10^6
13-HPOD	11 ± 2	99 ± 3	9.0×10^6
13-HPOT	4 ± 2	21 ± 2	5.3×10^6

Assays were performed at 30 °C in 1 ml 0.05 M sodium phosphate buffer, pH 7.0. Assays using the 9-hydroperoxy fatty acids as substrate contained 30 ng of purified protein. Assays using the 13-hydroperoxy fatty acids as substrate contained 60 ng of purified protein. Values represent the average ±SD of two experiments using independent protein preparations.

(820 s^{-1}) for 9-HPOD was comparable to turnover rates reported for other recombinant CYP74 enzymes (Song and Brash, 1991; Matsui et al., 2000; Itoh and Howe, 2001; Tijet et al., 2001).

Tissue specific expression of LeAOS3 in roots

EST sequencing data (www.tigr.org/tdb/lgi/) indicated that all ESTs corresponding to *LeAOS3* were identified in cDNA libraries constructed from either germinating seedlings (9 of 10 ESTs) or roots (1 of 10 ESTs). To further investigate the developmental expression of *LeAOS3*, RNA blot analysis was used to determine the abundance of *LeAOS3* transcript in various tomato organs. The results showed that *LeAOS3* mRNA accumulated in roots of mature plants with no expression detected in aerial tissues including cotyledons, leaves, stems, and flower buds (Figure 2.8). We also confirmed that *LeAOS3* was expressed early after seed germination (4 days after seed imbibition), when the radical had just emerged from the seed coat (data not shown). These findings indicate that *LeAOS3* transcript accumulation is tightly regulated by developmental cues and further suggest that expression of the gene is restricted to soil-exposed tissues.

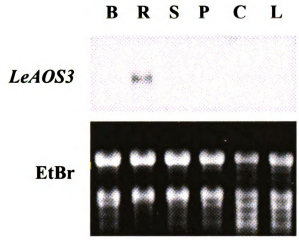

Figure 2.8 Tissue specific expression of *LeAOS3*. Total RNA was extracted from unopened flower buds (B) from six-week-old tomato plants and from roots (R), stems (S), petioles (P), cotyledons (C), and leaves (L) from 18-day-old plants. RNA blot was hybridized to a *LeAOS3* cDNA probe. An ethidium bromide (EtBr) stained agarose gel of the same samples is also shown.

Discussion

In the present study we report the functional characterization of a novel tomato cDNA (*LeAOS3*) encoding an AOS that catalyzes the production of ketol and cyclopentenone oxylipins from both 9- and 13-hydroperoxy fatty acids. Several features of the enzyme are distinct from previously characterized AOSs involved in JA biosynthesis. First, the deduced amino acid sequence of LeAOS3 is more similar to cucumber and melon HPLs than it is to 13-AOSs from tomato (*i.e.* LeAOS1 and LeAOS2) or other plants. In this context, LeAOS3 represents the first example of an AOS that is classified as a CYP74C; all other AOSs are classified as CYP74As. Second, in contrast to the specificity of most AOSs for 13-hydroperoxides, LeAOS3 exhibits a marked preference for 9-hydroperoxides. A similar substrate preference was reported for the two cucurbit HPLs that are the closest known relatives of LeAOS3 (Matsui et al., 2000; Tijet et al., 2001). This observation suggests that the sequence relatedness between various CYP74 P-450s is an indicator of substrate specificity. Consistent with this notion, CYP74Cs are more closely related to the 9-hydroperoxide-specific CYP74Ds than they are to members of the CYP74A and B subfamilies, which have relative specificity for 13-hydroperoxides. A third unique feature of *LeAOS3* is its tissue-specific expression pattern in germinating seeds and roots of mature plants. This finding supports previous studies showing that cell-free extracts from tomato roots catalyze the formation of α-ketols via the 9-LOX/AOS pathway (Caldelari and Farmer, 1998). Taken together, our results strongly suggest that LeAOS3 defines a class of AOSs that is distinct from those involved in JA biosynthesis in photosynthetic tissues. This idea is consistent with the notion that

oxylipin metabolism is organized into 9-LOX and 13-LOX pathways, each of which use a specialized type of AOS for production of distinct oxylipins in specific cell and tissue types (Howe and Schilmiller, 2002).

The overall characteristics of LeAOS3 suggest a biochemical function in the production of one or more 9-hydroperoxide derived oxylipins. Given the expression of *LeAOS3* in roots, it is conceivable that the enzyme plays a defensive role against soil-borne pests that affect roots or juvenile tissues (*e.g.* radical) as they emerge from the germinating seed. This hypothesis is in keeping with increasing evidence for a role of the 9-LOX pathway in plant defense against pathogens (Masui et al., 1989; Rance et al., 1998; Rusterucci et al., 1999; Weber et al., 1999; Gobel et al., 2001; Stumpe et al., 2001). However, whereas pathogen-induced stimulation of the 9-LOX pathway has been shown to activate the DES, epoxy alcohol synthase, and reductase branches of the 9-LOX pathway (Gobel et al., 2001), there is little evidence that biotic stress activates the 9-AOS pathway. A second possibility is that LeAOS3 catalyzes the production of oxylipins that play a role in plant development. Support for this hypothesis comes from two recent studies. One study provided evidence that 9-hydroxy-10-oxo-12,15-octadecadienoic acid, the α-ketol produced by the action of LeAOS3 on 9-HPOT, functions as a signal for flower development in *Lemna paucicostata* (Yokoyama et al., 2000). Another study showed that transgenic potato plants depleted in the expression of a *9-LOX* gene exhibited abnormal tuber development (Kolomiets et al., 2001). Although specific oxylipins that account for the tuber phenotype were not identified, the presence of 9-AOS activity in potato stolons (Hamberg, 2000) is consistent with a role for 9-hydroperoxide-derived compounds in tuber development.

One of the more interesting features of LeAOS3 is its involvement n the formation of 10-OPEA and 10-OPDA from 9-hydroperoxides of linoleic and linolenic acids, respectively. These novel cyclopentenones were recently identified as products of the 9-LOX/AOS pathway in potato (Hamberg, 2000). Indeed, several similarities between LeAOS3 and the potato stolon 9-AOS activity strongly suggest that the enzymes are functionally equivalent. First, the relative abundance of 10-OPEA, α-ketol, and γ-ketol products formed by the action of LeAOS3 on 9-HPOD was comparable to that reported for the potato enzyme. Second, the tissue-specific expression of *LeAOS3* is similar to that of the cyclopentenone-forming activity in potato, which was highest in roots and not detectable in leaves. A third similarity between LeAOS3 and the potato 9-AOS was the relatively high proportion of cyclopentenone product (*i.e.* 10-OPEA) formed from 9-HPOD. This feature of 9-AOS-catalyzed metabolism of hydroperoxy dienoic fatty acids contrasts other studies showing that non-enzymatic cyclization of allene oxides requires the presence of a double bond in the β,γ-position relative to the epoxide group (Vick and Zimmerman, 1979; Hamberg and Fahlstadius, 1990; Grechkin, 1994).

The formation of cyclopentenones by LeAOS3 is particularly interesting in light of the signaling activity exhibited by cyclopentenones (*e.g.* 12-OPDA) produced from the 13-LOX/AOS pathway. Recent studies (Stintzi et al., 2001) in Arabidopsis have shown that 12-OPDA can activate the expression of defense-related genes via a signal transduction pathway involving COI1, an F-box protein that is required for JA-mediated signaling (Turner et al., 2002). There is also evidence to suggest that the α,β-unsaturated carbonyl group located in the cyclopentenone ring 12-OPDA is involved in defense gene

activation via a COI1-independent signaling pathway (Stintzi et al., 2001). Such a signaling mechanism has been documented for cyclopentenone prostaglandins in which the reactive α,β-unsaturated carbonyl mediates conjugate addition to various intracellular targets (Straus and Glass, 2001). Given the structural similarity of 9-AOS-derived cyclopentenones to 12-OPDA (Figure 2.1), including the presence of an α,β-unsaturated carbonyl group in the cyclopentenone ring, a role for these compounds in signaling can be hypothesized. Additional work is needed to determine whether 9-AOS catalyzes the formation of 10-OPEA and 10-OPDA in plant tissues.

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Chapter 3

Regulation of Allene Oxide Synthase3 Expression in Tomato

Abstract

Allene oxide synthase3 (LeAOS3) is a CYP74 cytochrome P450 from tomato that metabolizes 9-hydroperoxides of linolenic and linoleic acid to unstable allene oxides that spontaneously convert in the presence of water to α- and γ-ketols or racemic cyclopentenones. This study focuses on understanding the regulation of *LeAOS3* expression and activity as a means to gain greater insight into the physiological function of the 9-AOS pathway. The expression of *LeAOS3* was highly induced in roots upon methyl-jasmonate (MeJA) treatment or wounding. Expression of *LeAOS3* in roots was abolished in the *jai1* mutant that is defective in jasmonic acid (JA) perception. Whereas expression of *LeAOS3* in roots requires a functional JA signaling pathway, expression in hypocotyls of germinating seedlings occurred constitutively and independently of JA, indicating that there are different tissue-specific modes of regulation for the 9-AOS pathway. Immunocytochemical studies showed that LeAOS3 protein is localized specifically in cortex cell layers of both hypocotyls and growing root tips. cDNA microarray analysis was used to identify other genes that are co-regulated with *LeAOS3* in tomato roots. These findings suggest a possible role for the 9-AOS pathway in defense against soil-borne invaders.

Introduction

Oxidative metabolism of polyunsaturated fatty acids gives rise to a diverse set of compounds collectively called oxylipins. Biosynthesis of oxylipins in plants is initiated by lipoxygenase (LOX), which adds molecular oxygen to the 9- or 13-position of C16 and C18 polyunsaturated fatty acids (Feussner and Wasternack, 2002). The hydroperoxide-containing fatty acids that are formed serve as substrates for the CYP74 family of cytochromes P450 that are responsible for generating different types of oxylipins (Figure 3.1) Several recent studies provide evidence that oxylipins play a role in a variety of developmental and stress-related processes (Howe and Schilmiller, 2002). The best example of this is the jasmonate family of oxylipins (collectively referred to as JAs) that includes jasmonic acid (JA) and its derivatives (Howe, 2005; Schaller et al., 2005). Whereas JAs are known to regulate many processes in plant development and defense, relatively little is known about the physiological function of other oxylipins.

LeAOS3 is a CYP74 cytochrome P450 that metabolizes 9-hydroperoxides of linolenic and linoleic acid to 9,10-epoxyoctadecatrienoic and 9,10-epoxyoctadecadienoic acids, respectively (Itoh et al., 2002). These unstable allene oxides spontaneously convert *in vitro* to α- and γ-ketols or racemic cyclopentenones (Figure 3.1). Several lines of evidence link the 9-LOX pathway to defense against pathogens. For example, oxylipin profiling studies have shown that several 9-LOX pathway products accumulate upon treatment with elicitors or pathogen infection (Gobel et al., 2001; Gobel et al., 2002). Consistent with these findings, antisense-mediated depletion of a 9-LOX in tobacco resulted in susceptibility to *Phytophthora parasitica* (Rance et al., 1998). Others have

Figure 3.1 CYP74-catalyzed oxylipin biosynthesis. Lipoxygenase (LOX) adds a hydroperoxy group at the 9- or 13-position of polyunsaturated fatty acids. The hydroperoxy fatty acids then serve as substrates for the CYP74 family of cytochromes P450 (in bold) that target the hydroperoxides to various branches of oxylipin biosynthesis. (DES, divinyl ether synthase; HPL, hydroperoxide lyase; AOS, allene oxide synthase; AOC, allene oxide cyclase; OPDA, 12-oxophytodienoic acid; OPR, OPDA-reductase)

Figure 3.1 CYP74-catalyzed oxylipin biosynthesis.

suggested a role for the 9-LOX pathway in the hypersensitive response to pathogen attack (Rusterucci et al., 1999; Jalloul et al., 2002). However, there is no evidence to date to indicate that the AOS branch of the 9-LOX pathway is involved in defense. An alternative hypothesis is that 9-AOS functions in plant development. Support for this idea comes from the observation that antisense suppression of a 9-LOX in potato (StLOX1) results in reduced tuber yield, and defective tuber development (Kolomiets et al., 2001). The presence of 9-AOS activity in potato roots, stolons, and tubers (Hamberg, 2000) indicates the possibility that 9-AOS could function in tuber development.

To gain additional insight into the function of the 9-LOX pathway, we studied the regulation and tissue-specific expression of *LeAOS3* in tomato. Analysis of a tomato mutant (*jai1*) disrupted in the ortholog of *Arabidopsis COI1* showed *LeAOS3* expression in roots requires JA perception. However, *LeAOS3* expression in hypocotyls of germinating seedlings was independent of COI1. Microarray analysis showed that the JA- and wound-induced expression of LeAOS3 in roots was very similar to that of other known defense genes. These advances in our understanding of the regulation of *LeAOS3* lay the foundation for testing specific hypotheses concerning the physiological role of the 9-LOX pathway in plants.

Materials and Methods

Plant materials and growth conditions

Tomato (*Lycopersicon esculentum*) cv Micro-Tom was used as the wild type for all experiments. Seeds were germinated on moist filter paper for experiments with germinating seedlings. Older plants were grown in Jiffy peat pots (Hummert International, Earth City, MO) for up to three weeks, and then transferred to a Baccto soil and sand mix (1:1; Michigan Peat Company, Houston, TX). Plants were maintained in a growth chamber under 17 h of light (200 $\mu E/m^2/s$) at 28°C and 7 h of dark at 18°C. Homozygous *jai1-1* mutants were obtained from a segregating F_2 population as described previously (Li et al., 2001). For methyl-JA (MeJA) treatment of tomato roots, plants growing in peat pots or pots with a soil and sand mix were watered with a 0.1 mM solution of MeJA. 500 ml of solution was added to a tray containing ~ 25 peat pots or 12 four-inch pots.

Gene expression analysis

RNA extraction and analysis was performed as previously described (Howe et al., 2000). A cDNA probe for *LeAOS3* was made from a full-length EST clone (cLEI16I12) obtained from Clemson University. As a loading control, identical RNA blots were hybridized with a cDNA probe for translation initiation factor *eIF4A* mRNA (EST clone cLED1D24). The quality of RNA was assessed by staining of agarose gels with ethidium bromide.

Antibody production and protein expression analysis

Recombinant His-tagged LeAOS3 was purified from *E. coli* as described previously (Itoh et al., 2002) and further purified by preparative SDS-PAGE. Acrylamide gel slices containing LeAOS3 were sent for polyclonal antibody production (Cocalico Biologicals, Reamstown, PA) following the company's standard protocol for immunization in rabbits. Total protein was extracted by grinding frozen plant tissue in liquid nitrogen and adding extraction buffer (50 mM sodium phosphate pH 7.0, 150 mM NaCl, 10% glycerol, and Roche mini-complete protease inhibitors) at a buffer to tissue ratio of approximately 2:1 (v/w). Plant debris was removed by centrifugation at 13,000 x g and the protein concentration of the supernatant was measured with the BCA assay (Pierce, Rockford, IL), with BSA as a standard. Protein was separated on 10% SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore, Bedford, MA) by standard procedures (Harlowe and Lane, 1988). Membranes were probed with anti-LeAOS3 serum (diluted 1:1000) in Tris-buffered saline (TBS) containing 1% (w/v) nonfat dry milk and 0.1% (v/v) Tween 20. Antigen-antibody complexes were detected with an alkaline phosphatase-conjugated secondary antibody (diluted 1:5000; Kirkegaard & Perry Laboratories, Gaithersburg, MD) in TBS containing 1% (w/v) nonfat dry milk and 0.1% (v/v) Tween 20, followed by detection with NBT/BCIP substrate (Roche, Indianapolis, IN).

LeAOS3 activity assay

LeAOS3 activity was measured as previously described with the following minor modifications (Caldelari and Farmer, 1998). Cell-free extracts were collected by grinding

tissue in a chilled mortar, transferring the tissue to a microcentrifuge tube and pelleting cell debris. The resulting supernatant was used immediately in the following activity assay. The reaction (total volume 0.4 ml) was initiated by addition of 15 µl of cell-free extract (~ 5 µg protein/µl) to 40 mM sodium phosphate buffer (pH 7.0) containing 10% glycerol and 0.56 µg of [1-^{14}C]-linoleic acid (50 µCi/µmol; Perkin Elmer, Boston, MA). After a 10 min incubation at 25°C, products were extracted with $CHCl_3$-MeOH (2:1) and the $CHCl_3$ phase was dried under a stream of nitrogen gas. Products were resuspended in 10 µl of $CHCl_3$ and separated by thin layer chromatography (TLC) using silica plates and petroleum ether/diethyl ether/formic acid (70:30:1) as the solvent system. Following separation, plates were analyzed by autoradiography. To generate oxylipin standards (hydroperoxy fatty acids, α-ketols, divinyl ethers), 9-LOX was partially purified from ripe tomato fruit pericarp as previously described (Smith et al., 1997). This enzyme was used alone or with purified recombinant LeAOS3 or LeDES (Itoh and Howe, 2001; Itoh et al., 2002) in the assay described above.

Immunocytochemistry

Root tissue (from four-week old plants grown in sand/soil mix) or hypocotyl tissue (from five-day old germinating seedlings) was fixed in FAA (3% w/v formaldehyde, 5% v/v acetic acid, 50% v/v ethanol) at room temperature for 3 h and embedded in polyester wax (Steedman, 1957) at 37°C overnight as previously described (Vitha et al., 2000). Tissue sections (9-µm-thick) were made with a Leica RM2155 microtome (Leica, Germany) and attached to poly-L-lysine coated glass slides (Sigma, St. Louis, MO). Sections were dewaxed and rehydrated as described (Vitha et al., 2000),

followed by an antigen retrieval procedure in which slides were submerged in 100 mM Tris-HCl (pH 10.0) in plastic Coplin jars and heated in a microwave to near boiling. The jars were allowed to cool at room temperature for 30 min and then washed once in PBS (0.14 M NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 3 mM NaN_3, pH 7.3) for 10 min. Slides were then transferred to blocking buffer (PBS containing 0.05% v/v Tween 20 and 2% w/v nonfat dry milk) for 2 h followed by incubation with purified LeAOS3 antibody (diluted 1:500) in blocking buffer overnight at room temperature. Control slides were incubated with an equivalent amount of pre-immune serum. After five 10 min wash steps in PBS containing 0.05% (v/v) Tween 20, slides were incubated for 30 min in blocking buffer followed by a 4 h incubation in blocking buffer with goat anti-rabbit secondary antibody conjugated with Alexa 488 fluorophore (diluted 1:300; Sigma). Slides were then washed four times (10 min each) in PBS containing 0.05% (v/v) Tween 20, then once for 10 min in PBS alone. After washing, 30 µl of mounting medium (1 mg/ml p-phenylendiamine in 0.01 M potassium phosphate (pH 7.4), 0.15 M NaCl, 90% glycerol; adjust pH to 8.0 with Tris-HCl (pH 8.0) and add DAPI to 1 µg/ml) was added to each slide. Slides were covered with a glass cover slip and sealed with nail polish. Sections were analyzed with an epifluorescence Zeiss Axio Imager M1 microscope (Zeiss, Germany) equipped with the appropriate filter set (excitation 472 nm, bandwidth 30 nm; emission 520 nm, bandwidth 35 nm).

To purify LeAOS3 antibodies, 225 µg of total protein from *jai1* roots, which do not express LeAOS3, was separated on a 10% polyacrylamide gel and transferred to an Immobilon-P membrane (see above). The membrane was blocked with 2% non-fat milk in PBS containing 0.05% (v/v) Tween 20 for 1 h, and then incubated with LeAOS3

antibodies (diluted 1:500) in blocking buffer for 1 h. LeAOS3 antibodies that remained in the blocking buffer was used directly for incubation with sectioned tissue on slides, or was stored at -80°C until needed.

Microarray analysis

Three-week-old wild-type and *jai1-1* tomato plants grown in peat pots were watered with a solution of 0.1 mM MeJA. At various times after treatment (1, 6, 12, and 24 h), roots from 10 plants were harvested, pooled, and frozen in liquid nitrogen. Roots from untreated plants were harvested as the "0" h time point. Three biological replicates were collected for each time point. For RNA extraction, tissue was ground under liquid nitrogen and transferred to a 13 ml polypropylene tube containing 4 ml phenol and 4 ml 100 mM Tris-HCl (pH 8.0) and then mixed by vortexing. After centrifugation, the aqueous phase was transferred to a new tube and extracted twice with 5 ml of chloroform. RNA was precipitated with LiCl (2M final concentration) overnight at 4°C, and then pelleted by centrifugation. The resulting RNA pellet was washed with cold 70% ethanol, air dried, and then resuspended in 300 µl of diethyl pyrocarbonate (DEPC)-treated water. RNA was then treated with RNase free DNaseI (Ambion, Austin, TX) according to the manufacturer's instructions. Following DNaseI treatment, RNA was extracted once with phenol/chloroform/isoamyl alcohol (25/24/1), and then precipitated overnight with 2.5 volumes of cold ethanol and 1/10 volume of 2.5 M sodium acetate (pH 4.8) at -20°C. RNA was collected by centrifugation and washed once with cold 70% ethanol, air dried, and then resuspended in DEPC-water. The quality of RNA was analyzed by gel-electrophoresis and quantified by UV absorbance.

All of the steps for microarray analysis (including cDNA production and labeling, hybridization, and data normalization with LOWESS) were performed by the TIGR Solanaceae Gene Expression profiling service according to their standard operating procedures (http://www.tigr.org/tdb/potato/microarray_SOPs.shtml). Version 3 of the TIGR potato cDNA microarray, which contains 15,264 potato cDNA clones with each clone spotted twice on the array (http://www.tigr.org/tdb/potato/microarray_comp.shtml), was used for all experiments. For our analysis, genes showing an expression ratio of ≥ 3 or ≤ 0.33 in at least two of the three biological replicates were counted as being differentially regulated. The final expression ratio was calculated as the average ratio from the three biological replicates.

Results

Wound-induced expression of LeAOS3 in roots requires the JA-signaling pathway

Wound-induced expression of *AOS* genes in leaves of several plants has been shown to involve the JA-signaling pathway (Laudert and Weiler, 1998; Howe et al., 2000; Maucher et al., 2000; Sivasankar et al., 2000; Ziegler et al., 2001). To determine whether JA signaling is involved in the regulation of *LeAOS3* expression, we treated wild-type and COI1-deficient *jai1* plants with a 0.1 mM solution of MeJA, and then collected root tissue at various times after treatment for RNA and western blot analysis. *LeAOS3* expression increased after 1 h of treatment and peaked at the 12 h time point (Figure 3.2A). Relative to mRNA levels, a delayed increase in LeAOS3 protein levels was observed 8 h roots after MeJA treatment (Figure 3.2B). In contrast to the wild-type

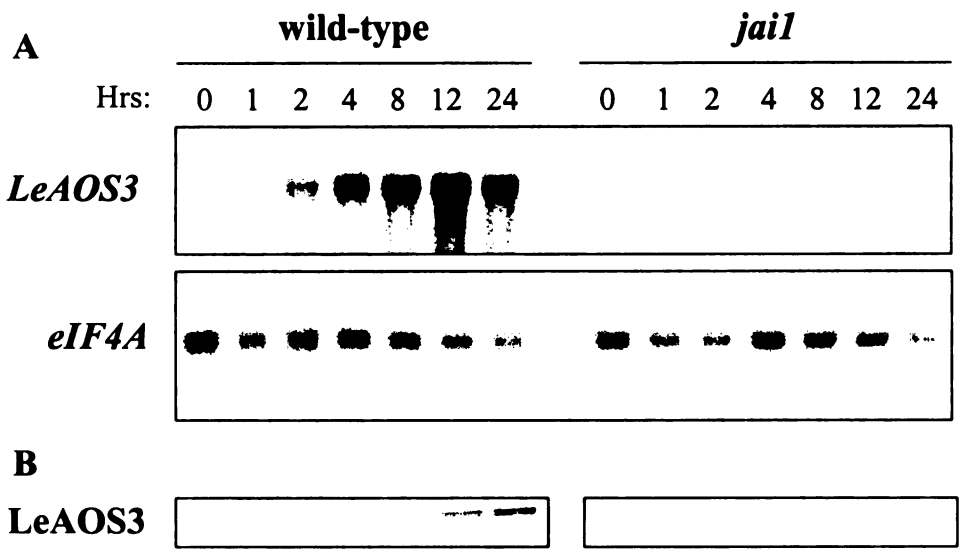

Figure 3.2 Accumulation of *LeAOS3* transcript and protein in response to MeJA. Four-week-old wild-type and *jai1* plants were treated with a solution of 0.1 mM MeJA and roots were collected at the indicated times after treatment for RNA or protein extraction. Identical RNA blots (A) were probed with either *LeAOS3* or *eIF4A* (loading control) cDNA probes. Western blots (B) were probed with antiserum raised against recombinant LeAOS3.

expression of *LeAOS3* was not detected in control or MeJA-treated *jai1* roots (Figure 3.2A). Likewise, LeAOS3 protein was undetectable in *jai1* roots (Figure 3.2B).

It is well established that wounding causes a rapid and transient increase in the accumulation of endogenous JA, which triggers the induction of JA-regulated genes (Schilmiller and Howe, 2005). Experiments were performed to determine whether wounding induces the expression of *LeAOS3* in roots. Roots of three-week old wild-type and *jai1* plants were wounded by squeezing with a hemostat, which resulted in crushing and breaking of root tissue. Western blot analysis showed that, similar to MeJA-treatment, wounding induced the accumulation of LeAOS3 protein in wild-type but not *jai1* plants (Figure 3.3A). To determine whether increased LeAOS3 protein levels were associated with increased LeAOS3 activity, we utilized a previously described assay to measure oxylipin production in cell-free extracts from the unwounded and wounded roots (Caldelari and Farmer, 1998). Incubation of unwounded wild-type root extract with [1-^{14}C]-linoleic acid (^{14}C-18:2) resulted in the production of several labeled compounds. One of these compounds co-chromatographed with the product formed after incubation of the same substrate with tomato 9-LOX (prepared from tomato fruit) and recombinant LeAOS3 (Figure 3.3B). Based on our previous finding that 9-hydroxy-10-oxo-(12,15)-octadecadienoic acid (α-ketol) is the major 9-hydroperoxy linoleic acid (9-HPOD) derived product formed by LeAOS3 *in vitro* (Itoh et al., 2002), this compound is likely the α-ketol (marked with arrow, Figure 3.3B). Cell-free extracts prepared from wounded wild-type roots catalyzed the formation of higher levels of the α-ketol compared to extracts from unwounded roots. This is consistent with the wound-induced accumulation of LeAOS3 protein. Extracts from *jai1* roots showed little or no capacity to synthesize the

Figure 3.3 Induction of LeAOS3 protein and activity after wounding.

Roots of four-week-old wild-type and *jai1* plants growing in peat pots were wounding by crushing with a hemostat. Roots were collected at various times for either protein extraction and western blotting with LeAOS3 antiserum (A) or LeAOS3 activity assays (B). For activity assays, cell-free extracts from root tissue was incubated with ^{14}C-linoleic acid followed by extraction of oxylipins and analysis by thin layer chromatography. As controls, 9-LOX activity partially purified from tomato fruit was used to generate ^{14}C-9-hydroperoxy linolenic acid standard (9-LOX). Co-incubation of 9-LOX with recombinant LeAOS3 (9-LOX + AOS3) produced 9-hydroxy,10-oxo-octadecadienoic acid standard (α-ketol, marked with arrow).

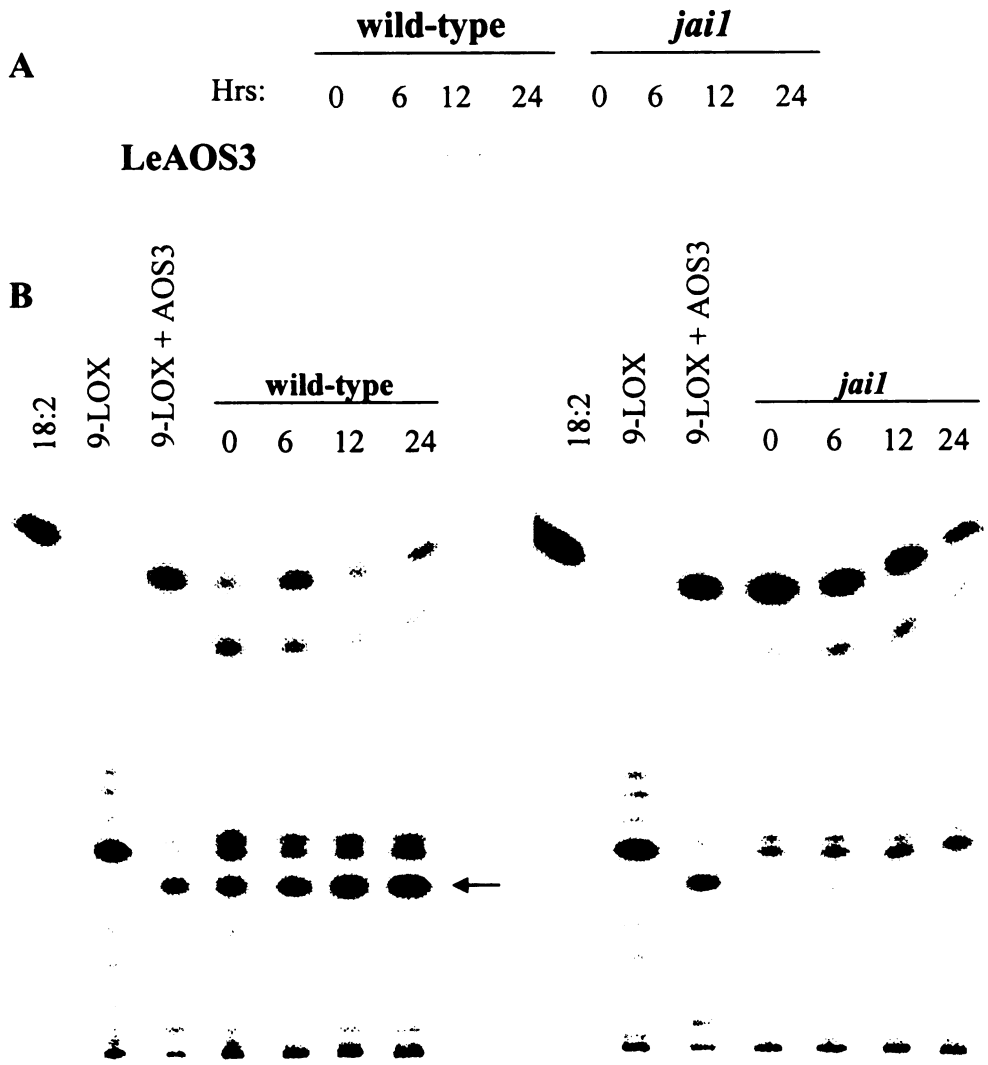

Figure 3.3 Induction of LeAOS3 protein and activity after wounding.

α-ketol from ^{14}C-18:2 (Figure 3.3B). The low level of co-migrating product formed by *jai1* extracts is likely to be 13-hydroxy-12-oxo-(9,15)-octadecadienoic acid, the α-ketol from the 13-AOS pathway, which migrates at the same position with this solvent system. These findings indicate that the expression of *LeAOS3* in roots is dependent on the JA-signaling pathway.

Expression of LeAOS3 in hypocotyls of germinating seedlings is independent of COI1

Analysis of *LeAOS3* EST data (www.tigr.org/tdb/lgi/plant.shtml) showed that all ESTs corresponding to *LeAOS3* were from cDNA libraries constructed from either root (1 of 12 ESTs) or germinating seedlings (11 of 12 ESTs). The relatively high abundance of ESTs in the germinating seedling library prompted us to investigate *LeAOS3* expression at this developmental stage. RNA blot analysis showed that *LeAOS3* in wild-type germinating seedlings was expressed to its highest levels four days after seed imbibition, and decreased substantially over the period of eleven days (Figure 3.4). Unlike in root tissue, *LeAOS3* expression in germinating *jai1* seedlings was only slightly decreased in comparison to the wild type (Figure 3.4). We also analyzed LeAOS3 protein levels in roots and germinating seedlings of wild-type and *jai1* plants. Consistent with RNA-blot analysis, LeAOS3 protein was detected in roots and germinating seedlings of both genotypes, while no LeAOS3 protein accumulated in *jai1* roots (Figure 3.5). LeAOS3 activity in extracts of wild-type and *jai1* germinating seedlings was measured as well. The major oxylipin formed from ^{14}C-18:2 extracts from wild-type seedlings co-chromatographed with the α-ketol produced by recombinant LeAOS3 (Figure 3.6). The amount of α-ketol produced with extracts from *jai1* was comparable to wild-type levels

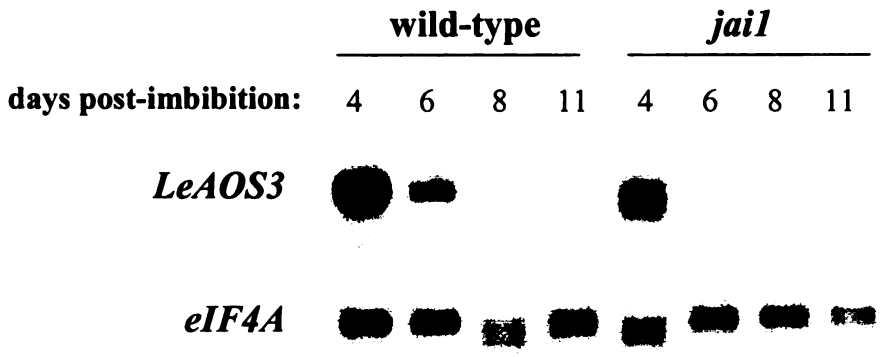

Figure 3.4 *LeAOS3* expression in germinating seedlings. Total RNA was extracted from wild-type and *jai1* seedlings germinated on wet filter paper for the indicated times. Identical blots were hybridized to either *LeAOS3* or *eIF4A* (loading control) cDNA probes.

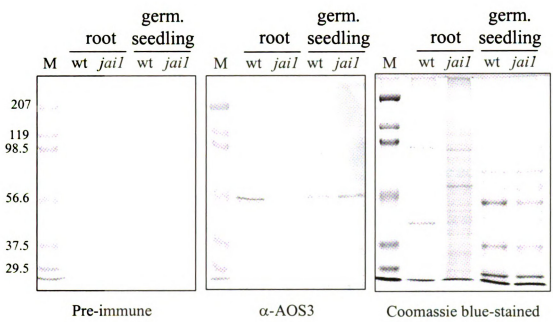

Figure 3.5 LeAOS3 protein levels in root and germinating seedlings.

Total protein was extracted from roots of soil grown six-week-old wild-type (wt) and *jai1* plants and from four-day-old seedlings germinated on wet filter paper. Proteins were separated by SDS-polyacrylamide gel electrophoresis (10% gels) and either stained with Coomassie blue or transferred to PVDF membranes and probed with antiserum raised against LeAOS3 or pre-immune serum. Numbers at left indicate sizes of molecular weight standards (M).

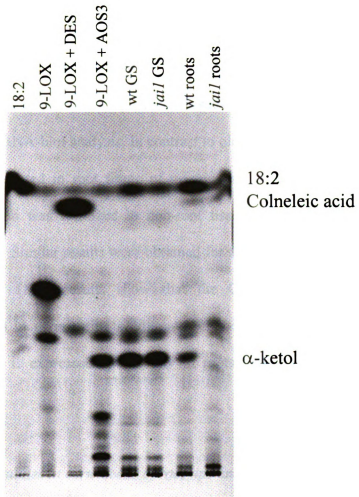

Figure 3.6 LeAOS3 activity in cell-free extracts of wild-type and *jai1*.

LeAOS3 activity was measured in extracts of 5-day old germinating seedlings (GS) and roots from 3-week old wild-type and *jai1* plants. In addition to controls described in the legend to Fig 3.3, a control for colneleic acid was made using partially purified 9-LOX with recombinant LeDES (9-LOX + DES).

(Figure 3.6). These results show that *LeAOS3* expression in germinating seedlings is not dependent on the JA-signaling pathway.

The COI1-independent expression of *LeAOS3* in germinating seedlings prompted us to determine the spatial pattern of *LeAOS3* expression within the seedling. Root or non-root (i.e. hypocotyl, seed coat, cotyledons) tissues from 5-day-old seedlings was collected and used for RNA-blot analysis. In contrast to older, soil-grown plants, *LeAOS3* expression was not detected in root tissue of germinating seedlings. At this stage of development, expression was localized to non-root tissue of the tomato germinating seedling (Figure 3.7A). Similar results were obtained for the analysis of LeAOS3 protein levels (Figure 3.7B). These results show that the COI1-independent constitutive expression of LeAOS3 in germinating seedlings occurs in non-root tissues whereas in older plants, LeAOS3 is expressed specifically in roots and is controlled by the JA signaling pathway.

LeAOS3 protein accumulates in cortex cells of root tips and hypocotyls

We used immunofluorescence microscopy to investigate the cell type-specific expression pattern of LeAOS3 in roots and hypocotyls of germinating seedlings. As a control for these experiments, we took advantage of the *jai1* mutant that lacks expression of LeAOS3 in root tissues. In hypocotyl cross-sections from wild-type germinating seedling, LeAOS3 protein was detected in the first layer of cortex cells, adjacent to the epidermal cell layer (Figure 3.8A' and A"). LeAOS3 protein was detected at lower levels in other cortex cell layers, but not in epidermal cells or central cell layers the comprise the vascular tissues. A similar pattern of expression was observed in cross-sections of

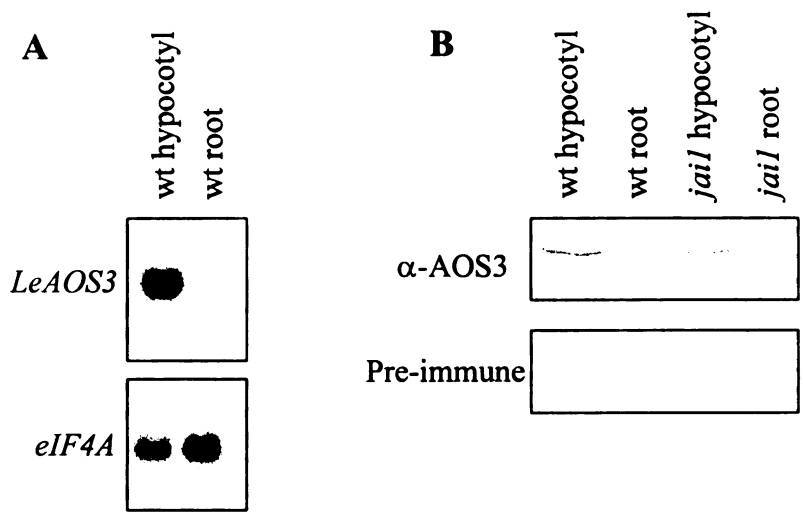

Figure 3.7 LeAOS3 is expressed in non-root tissues of germinating seedlings.

(A) Five-day-old wild-type germinating seedlings were dissected into root segments and the remaining non-root tissues (labeled as hypocotyl) for RNA-blot analysis of *LeAOS3* expression.

(B) Wild-type and *jai1* (5-day-old) germinating seedlings were dissected into root and hypocotyl segments and total protein was extracted from the individual tissues. Shown are western blots probed with LeAOS3 antibodies or pre-immune serum.

MeJA-treated wild-type root tips (Figure 3.8B' and B"). Longitudinal sections of this tissue showed that LeAOS3 protein expression is highest in the cortex layer near the tip, and decreases as cells elongate and mature (Figure 3.8C' and C"). This expression pattern closely resembles that of a subset of JA-responsive genes expressed in *Arabidopsis* roots (Birnbaum et al., 2003). In sections incubated with pre-immune serum (Figure 3.8D') or in sections of *jai1* roots incubated with LeAOS3 antibodies (Figure 3.8E'), no signal was detected. In older root tissues, weak expression was seen in the cortex cells. Signal in the epidermal cells was also seen in sections incubated with pre-immune serum, and thus was non-specific labeling. High expression was observed in sections through a lateral root, which showed LeAOS3 protein accumulating in the root tip (Figure 3.8F' and F").

MeJA-induced gene expression in tomato roots

To gain additional insight into the function of LeAOS3, we used microarray analysis to identify genes that are co-regulated with LeAOS3. The microarray used in these experiments was a *Solanum tuberosum* (potato) cDNA array constructed at The Institute for Genome Research (TIGR). Hybridizations performed with RNA from various Solanaceous species have previously established a high degree of genomic similarity throughout the family and validated the use of the potato array to study gene expression in tomato (see hybridization data available on-line at http://www.tigr.org/tdb/potato/SGED_index2.shtml). Our experimental set-up was to compare expression in wild-type and *jai1* roots at various times (1, 6, 12, and 24 h) after treatment with MeJA, as well as untreated controls. Of 11,512 sequenced verified cDNAs on the array, 272 were identified as being regulated by MeJA in at least one time-point

Figure 3.8 Immunolocalization of LeAOS3 protein. Hypocotyls from 5-day-old germinating seedlings, or roots from 3-week-old plants, were used for immunolabeling of LeAOS3. **A-G,** bright-field images; **A'-G',** immunofluorescence of same section shown in bright-field image. **A-C, E, F** were incubated with antibodies raised against LeAOS3, **D** & **G** were incubated with pre-immune serum. **A** cross section of untreated wild-type hypocotyl. **B, D** cross section of MeJA-treated wild-type root tip. **C**, longitudinal section of MeJA-treated wild-type root tip. **E**, cross section of MeJA-treated *jai1* root tip. **F, G** cross section of MeJA-treated wild-type root near root-shoot junction.

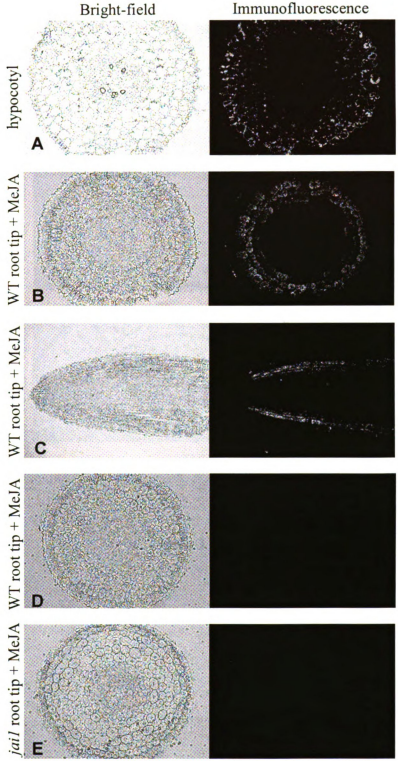

Figure 3.8 Immunolocalization of LeAOS3 protein

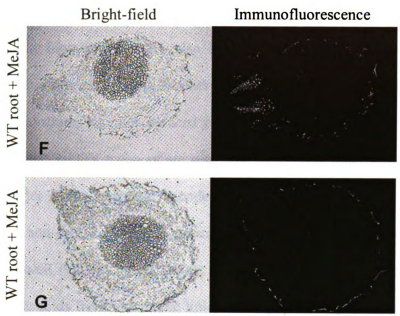

Figure 3.8 (cont'd)

(expression ratio ≥ 3 or ≤ 0.33, wt vs. *jai1*; Table 3.1). As seen for JA-regulated genes in leaves (Schmidt et al., 2005), most of the identified genes in roots were up-regulated (235) rather than down-regulated by the JA-signaling pathway (37). Several known JA-regulated defense genes were found to be induced, including those encoding proteinase inhibitors I and II, polyphenol oxidase, and cystatin (Zhao et al., 2003). Several genes involved in oxylipin biosynthesis were also highly induced in a manner similar to *LeAOS3*. Divinyl ether synthase (DES), another CYP74 enzyme that uses 9-hydroperoxy fatty acids as its substrate, was not induced by MeJA treatment, indicating that only certain branches of the 9-LOX pathway are regulated by JA-signaling. The most highly expressed genes after MeJA-treatment encoded patatin isoforms. Patatins are storage proteins that have non-specific lipolytic activity and have been implicated as having defensive roles as well (Dhondt et al., 2000; Sharma et al., 2004). It is possible that patatins release fatty acids from membrane lipids for oxylipin biosynthesis during times of stress.

Table 3.1 MeJA-regulated genes in tomato roots. Three-week-old wild-type and *jai1* plants were watered with 0.1 mM MeJA and roots were harvested for RNA extraction at the indicated times. Roots from untreated plants were collected as the "0" h time point. Microarray analysis was performed using the TIGR potato array (see text for details). Genes that were up-regulated ≥ 3-fold or ≤ 0.33-fold in wild-type at any time point are listed, with the potato clone number and annotations given as well as the expression ratios at each time point (sorted on the 24 h time point). A ratio of "0.00" indicates flagged spot for which data was not reliable.

Table 3.1 MeJA-regulated genes in tomato roots

Clone	Description	Expression ratios (wt/*jai1*) 0 h	1 h	6 h	12 h	24 h
STMHY52	Patatin class I precursor (Potato tuber protein). {Solanum tuberosum}	4.17	5.27	52.69	225.35	316.63
STMHU29	class I patatin - potato {Solanum tuberosum}	2.19	2.99	31.91	132.47	233.15
STMHL62	Patatin T5 precursor (Potato tuber protein). {Solanum tuberosum}	2.01	3.48	25.72	126.23	190.43
STMGN35	Patatin T5 precursor (Potato tuber protein). {Solanum tuberosum}	2.10	3.30	21.11	97.67	183.71
STMHA69	patatin precursor non-sucrose-inducible - {Solanum brevidens}	0.00	2.96	17.99	93.20	144.09
STMGD48	Patatin T5 precursor (Potato tuber protein). {Solanum tuberosum}	2.76	2.66	18.39	111.77	128.48
STMHT35	proteinase inhibitor II {Solanum tuberosum}	0.89	1.74	12.84	52.42	117.49
STMGZ27	(Q9SLQ1) EEF53 protein (Fragment)	4.26	14.10	94.35	104.87	117.11
STMHN37	(Q9LM55) F2E2.11	1.12	2.57	28.73	72.09	98.10
STMGG43	class I patatin - potato {Solanum tuberosum}	1.75	2.08	11.49	63.22	93.56
STMHR92	(Q8L8N8) 70kD heat shock protein	1.30	1.99	10.92	55.93	76.53
STMCF50	(Q8RZ46) Lipase-like protein	3.75	8.14	50.78	71.67	72.47
STMGP96	catechol oxidase (allele POT33) - potato {Solanum tuberosum} (EC 1.10.3.1)	4.35	7.20	27.16	61.04	70.16
STMHQ63	(Q7PWX9) EbiP4168 (Fragment)	0.00	0.00	9.94	36.28	60.40
STMGS46	(Q9FR53) PTOR	3.24	7.99	39.11	71.10	59.02
STMCI55	Polyphenol oxidase B chloroplast precursor {Lycopersicon esculentum}	3.91	7.83	32.49	61.08	54.29
STMHE94	(Q9FRP6) Putative ubiquinone oxidoreductase subunit	1.06	1.81	14.10	34.31	49.90
STMCE83	extensin homolog - potato (fragment) {Solanum tuberosum}	3.42	8.68	26.23	47.35	46.58
STMCI79	extensin homolog - potato (fragment) {Solanum tuberosum}	2.27	6.40	30.52	50.06	46.22
STMEB19	Polyphenol oxidase A chloroplast precursor {Lycopersicon esculentum}	3.65	8.19	33.89	60.69	45.60
STMCK27	(Q43648) Proteinase inhibitor I	0.00	0.83	3.48	19.70	44.85
STMGA58	(Q8GU21) Orcinol O-methyltransferase (Fragment)	1.71	3.33	16.03	34.76	42.42
STMEB67	Polyphenol oxidase A chloroplast precursor {Lycopersicon esculentum}	3.89	8.17	36.94	60.43	41.99
STMDB78	(P58602) Cysteine protease inhibitor 4 (PCPI-23)	1.12	1.18	4.78	10.71	41.23
STMCR06	extensin homolog - potato (fragment) {Solanum tuberosum}	2.95	5.21	18.72	43.24	38.93
STMGR43	nucleoporin-like protein [imported] - Arabidopsis thaliana {Arabidopsis thaliana}	1.04	1.76	5.85	18.82	37.33
STMCC57	Proteinase inhibitor type II CEVI57 precursor. {Lycopersicon esculentum}	0.00	1.19	3.50	13.12	34.07
STMGE39	(Q9SLQ1) EEF53 protein (Fragment)	2.17	6.09	28.19	34.83	33.84
STMCO50	(Q43651) Proteinase inhibitor I (Fragment)	0.96	0.77	2.11	13.84	31.82
STMDU79	catechol oxidase (allele POT32) - potato {Solanum tuberosum} (EC 1.10.3.1)	3.80	6.95	23.97	41.45	31.31
STMDO41	catechol oxidase (allele POT32) - potato {Solanum tuberosum} (EC 1.10.3.1)	3.62	7.15	21.50	33.32	30.53

Table 3.1 (cont'd)

Clone	Description	Expression ratios (wt/*jai1*) 0 h	1 h	6 h	12 h	24 h
STMHI30	unknown protein {Arabidopsis thaliana}	1.15	1.53	4.53	14.69	29.57
STMHN34	(Q948G6) Putative epsin	1.06	1.39	4.24	11.81	28.82
STMGE45	(Q9FKL5) Adenosylmethionine-8-amino-7-oxononanoate aminotransferase-like protein	2.38	3.77	15.67	42.10	28.42
STMHP08	(Q84ZV5) Polyprotein	0.93	1.42	4.44	14.44	27.70
STMCQ55	Aspartic protease inhibitor 10 precursor (Wound-induced aspartateproteinase CDI inhibitor).	1.59	1.24	4.35	16.02	27.46
STMIP09	(O48552) Copper amine oxidase precursor (EC 1.4.3.6)	1.22	1.68	9.40	23.32	27.01
STMGG85	transferase family protein, similar to deacetylvindoline 4-O-acetyltransferase	3.06	7.98	18.61	28.10	26.61
STMCS50	extensin homolog - potato (fragment) {Solanum tuberosum}	1.84	3.13	15.52	32.42	26.49
STMHA70	hypothetical protein T18I24.10 [imported] - Arabidopsis thaliana	1.01	1.07	2.28	14.80	25.45
STMCX33	Cysteine protease inhibitor 1 precursor (PCPI 8.3) (P340) (P34021). {Solanum tuberosum}	0.00	0.00	2.16	9.33	24.93
STMHQ48	WD-40 repeat regulatory protein tup1 homolog - Arabidopsis thaliana	0.95	1.37	5.31	12.46	24.71
STMGY25	Proteinase inhibitor I precursor. {Solanum tuberosum}	1.19	1.03	2.95	15.41	24.65
STMJF07	lipoxygenase LX-3 - potato {Solanum tuberosum} (EC 1.13.11.12 EC 1.13.11.12)	1.57	3.71	17.03	26.62	23.83
STMJK87	(Q43190) Lipoxygenase (EC 1.13.11.12)	2.08	4.79	22.14	24.75	23.68
STMCY27	(Q9LU88) Acetyltranferase-like protein	3.62	8.99	16.59	30.09	23.21
STMIU61	(Q43651) Proteinase inhibitor I (Fragment)	1.04	0.94	2.96	11.62	23.17
STMDJ96	(Q9AXP7) PnFL-2	6.38	14.04	12.20	21.86	23.14
STMGN54	(Q7XMY0) OSJNBb0006L01.9 protein	1.25	1.71	3.32	13.10	22.83
STMIP69	(Q8LAE1) Subtilisin-like serine protease	2.09	3.17	5.24	16.70	22.12
STMCC47	(Q94I88) Putative miraculin (Fragment)	0.97	0.88	1.82	11.38	21.66
STMDP28	(Q9LVI4) Gb|AAF09073.1 (AT3g17860/MEB5_8)	3.46	8.91	20.09	19.11	21.27
STMIX49	(Q9SE08) Cystatin	1.11	1.14	3.35	11.20	20.21
STMCX74	(Q8W2K6) Proteinase inhibitor IIa	1.16	1.28	2.29	8.17	20.19
STMGW67	(Q86Z93) Kinesin	1.06	1.26	3.23	12.36	19.94
STMCP56	Polyphenol oxidase B chloroplast precursor (Fragment).	2.00	4.77	10.51	19.58	19.91
STMHK02	(Q9FI45) Carbonyl reductase-like protein	1.10	1.25	2.94	10.67	18.75
STMCQ37	(Q9FFF6) Leucoanthocyanidin dioxygenase-like protein (AT5g05600/MOP10_14)	2.88	24.71	24.03	30.81	18.65
STMDB46	Polyphenol oxidase B chloroplast precursor (Fragment).	2.70	5.57	14.24	24.26	18.40
STMCZ36	(BAD04851) Hypothetical protein PPS3	3.47	7.26	15.09	14.54	16.58
STMCL26	Cysteine protease inhibitor 1 precursor (PCPI 8.3) (P340) (P34021). {Solanum tuberosum}	1.50	0.93	2.31	9.11	16.51
STMJP82	(BAD04851) Hypothetical protein PPS3	3.34	8.10	15.78	15.37	16.41

Table 3.1 (cont'd)

Clone	Description	Expression ratios (wt/*jai1*) 0 h	1 h	6 h	12 h	24 h
STMGW66	(Q84S34) Short chain alcohol dehydrogenase-like protein	1.32	3.09	8.76	21.68	16.21
STMHH45	(Q9FYX3) BAC19.3	1.21	1.34	2.71	9.55	15.51
STMGF92	(BAC83154) Putative CMP-sialic acid transporter	1.23	1.14	1.77	8.80	15.47
STMHG27	probable nonsense-mediated mRNA decay protein [imported] - Arabidopsis thaliana	1.02	1.21	2.90	5.43	14.24
STMHQ67	Cysteine protease inhibitor 1 precursor (PCPI 8.3) (P340) (P34021). {Solanum tuberosum}	1.10	0.00	2.09	9.87	14.23
STMDG64	(Q9LVE9) Genomic DNA chromosome 3 P1 clone: MIL23	2.25	3.61	11.48	27.12	13.76
STMGJ53	unknown protein {Oryza sativa (japonica cultivar-group)}	1.66	2.06	10.42	10.65	13.72
STMGO76	(Q7XJ92) Poly(A) polymerase	0.96	1.17	2.00	7.81	13.66
STMHH43	(Q9ZR69) Acetyl-CoA synthetase (EC 6.2.1.1)	0.95	1.20	3.35	7.48	13.19
STMCN05	probable lipoxygenase (clone H1) - potato {Solanum tuberosum} (EC 1.13.11.12)	0.00	1.23	0.00	1.97	13.12
STMDU35	(Q8VWQ1) Dehydration-induced protein RD22-like protein	2.36	3.57	9.71	23.47	12.88
STMCK80	(Q94IC5) BURP domain-containing protein (Fragment)	2.23	3.55	9.98	25.98	12.69
STMHW35	(Q9FK32) Similarity to unknown protein	1.97	4.50	9.91	11.58	12.45
STMGL30	(Q8VWQ1) Dehydration-induced protein RD22-like protein	1.92	4.03	10.32	16.41	12.41
STMDD94	(Q84S34) Short chain alcohol dehydrogenase-like protein	0.00	2.87	12.44	24.41	12.31
STMGR18	(Q940F7) Hypothetical protein	0.00	1.44	3.68	7.97	12.27
STMCY06	unknown {Arabidopsis thaliana}	2.23	3.48	10.18	25.36	12.23
STMGS72	cysteine-rich extensin-like protein 2 precursor - common tobacco {Nicotiana tabacum}	1.24	2.35	6.56	10.79	11.76
STMGB05	(Q9LIY3) Hypothetical protein	2.17	3.24	6.47	10.99	11.71
STMGV14	(Q8VWQ1) Dehydration-induced protein RD22-like protein	2.15	3.81	10.89	23.16	10.87
STMGU77	extensin homolog - potato (fragment) {Solanum tuberosum}	1.21	1.99	4.86	9.80	10.55
STMHJ26	(Q94IC5) BURP domain-containing protein (Fragment)	1.96	3.39	8.50	16.81	10.11
STMGW82	(Q9LDU5) F28G4.16 protein (F1L3.3) (Expressed protein)	2.82	10.59	12.03	8.92	9.32
STMGT86	(Q94IC5) BURP domain-containing protein (Fragment)	2.00	3.18	8.28	18.54	9.32
STMGP14	At1g64600 {Arabidopsis thaliana}	1.30	2.01	3.68	6.91	9.14
STMGW17	(Q9LHT0) Short chain alcohol dehydrogenase-like	1.86	3.61	10.08	10.99	9.12
STMIW94	IAA-amino acid hydrolase 3 precursor. {Arabidopsis thaliana}	0.00	20.97	10.87	12.77	8.92
STMGB30	(Q8VWQ1) Dehydration-induced protein RD22-like protein	2.15	2.41	7.78	12.86	8.77
STMCV17	(Q93X16) Spermidine synthase (EC 2.5.1.16)	1.29	2.45	7.07	8.06	8.55
STMGW11	(Q93XI2) BURP domain-containing protein (Fragment)	1.94	2.37	5.91	13.20	8.46
STMGM04	(Q93XI2) BURP domain-containing protein (Fragment)	2.24	3.31	7.61	15.64	8.36

Table 3.1 (cont'd) Expression ratios (wt/*jai1*)

Clone	Description	0 h	1 h	6 h	12 h	24 h
STMCX10	cytochrome P450-like protein - Arabidopsis thaliana	3.14	25.99	24.75	22.49	8.24
STMHH08	(AAQ04608) Valencene synthase	1.38	2.27	5.11	5.02	8.06
STMCO57	(Q86AQ6) Similar to Mus musculus (Mouse). formin 1 isoform IV (Limb deformity protein)	2.58	3.13	7.14	8.50	7.65
STMGG72	fibroblast activation protein {Mus musculus}	1.07	1.05	1.57	4.03	7.57
STMGL87	(Q94IC5) BURP domain-containing protein (Fragment)	1.98	3.16	7.56	14.90	7.45
STMGS04	(Q8GUQ8) Xanthine dehydrogenase 1 (EC 1.1.1.204)	1.44	1.89	5.57	6.57	7.42
STMIV77	(AAQ20041) Isoflavone 3'-hydroxylase (Fragment)	1.54	2.45	5.03	7.65	7.37
STMCC64	protein BYJ15 - common tobacco (fragment) {Nicotiana tabacum}	2.47	6.44	8.51	10.53	7.31
STMHT13	(Q9FHA8) Genomic DNA chromosome 5 TAC clone:K21H1	1.55	9.30	8.56	9.88	7.30
STMCV86	(BAD04851) Hypothetical protein PPS3	1.99	3.06	7.33	5.75	7.10
STMCH16	(Q8L5K5) Ovule/fiber cell elongation protein Ghfe1	2.47	3.04	6.71	8.04	6.79
STMHZ55	(Q9AXU0) Major latex-like protein	1.05	1.12	3.61	5.04	6.67
STMEA65	(Q9M4X2) Putative cytochrome P450	1.92	2.78	6.86	7.09	6.55
STMDV35	Zeatin O-glucosyltransferase (Trans-zeatin O-beta-D-glucosyltransferase). {Phaseolus lunatus}	2.32	2.82	6.71	8.02	6.49
STMCN23	(O82681) Lanatoside 15'-O-acetylesterase precursor	1.31	2.03	4.65	6.60	6.41
STMCY29	Zeatin O-glucosyltransferase (Trans-zeatin O-beta-D-glucosyltransferase). {Phaseolus lunatus}	2.31	2.73	7.10	8.48	6.38
STMDD32	Zeatin O-glucosyltransferase (Trans-zeatin O-beta-D-glucosyltransferase). {Phaseolus lunatus}	2.17	3.03	6.70	8.00	6.30
STMHL80	Yippee-like protein At4g27740. {Arabidopsis thaliana}	0.00	1.16	0.00	4.62	6.25
STMDD15	(Q8RXA4) Putative glucosyltransferase	2.38	3.56	6.76	8.48	6.01
STMFB13	(Q84LG0) 5-Epl-aristolochene synthase	0.00	1.86	3.34	5.80	6.00
STMDV29	(O22616) Ornithine decarboxylase (EC 4.1.1.17)	1.53	2.71	4.99	6.66	5.82
STMCF04	(Q8L5K5) Ovule/fiber cell elongation protein Ghfe1	2.54	3.28	6.98	7.02	5.80
STMCZ02	acyl CoA reductase-like protein - Arabidopsis thaliana	1.96	2.06	5.25	6.91	5.73
STMCY65	(O82676) 1-deoxyxylulose 5-phosphate synthase	2.02	2.73	5.71	8.55	5.70
STMCF39	(Q9ZSH4) 2-oxoglutarate-dependent dioxygenase	2.21	2.25	6.01	6.58	5.63
STMCL06	(Q9ZSH4) 2-oxoglutarate-dependent dioxygenase	2.27	2.27	5.46	7.14	5.63
STMGI29	(Q9ZSH4) 2-oxoglutarate-dependent dioxygenase	2.11	2.24	4.56	5.64	5.51
STMCN85	(Q9ZSH4) 2-oxoglutarate-dependent dioxygenase	2.59	2.46	5.17	7.83	5.51
STMCU25	bHLH transcription factor {Arabidopsis thaliana}	1.80	5.06	5.34	6.49	5.50
STMJI30	(Q9LMI6) T2D23.3 protein	1.54	2.06	0.00	11.00	5.40
STMHA17	(Q7XAA4) WRKY12	1.10	1.04	1.76	3.32	5.40

Table 3.1 (cont'd)

Clone	Description	Expression ratios (wt/*jai1*) 0 h	1 h	6 h	12 h	24 h
STMCL01	carbonate dehydratase chloroplast - common tobacco (fragment)	1.28	0.33	1.92	4.08	5.38
STMGN22	unknown protein {Arabidopsis thaliana}	1.03	1.20	1.76	3.26	5.36
STMCP52	(O22616) Ornithine decarboxylase (EC 4.1.1.17)	1.46	2.51	4.43	5.80	5.26
STMCQ79	(Q84P53) Gamma-aminobutyrate transaminase subunit isozyme 2 (EC 2.6.1.19)	1.80	1.83	4.47	4.32	5.16
STMGG24	unknown {Arabidopsis thaliana}	1.16	1.03	1.13	3.09	5.15
STMCQ65	(Q9LWK9) Genomic DNA chromosome 1 PAC clone:P0485D09	1.32	1.40	3.63	4.07	5.08
STMCP75	S-adenosylmethionine decarboxylase {Solanum tuberosum} (EC 4.1.1.50)	1.17	1.05	2.64	3.76	5.02
STMGM68	(O82238) At2g47690 protein	1.12	1.18	1.49	2.90	5.01
STMHF49	(Q8VWQ1) Dehydration-induced protein RD22-like protein	1.71	2.41	5.86	5.88	4.98
STMHO27	(AAQ65188) At3g26950	1.07	0.92	0.97	1.80	4.82
STMGG42	Cell elongation protein DIMINUTO (Cell elongation protein Dwarf1). {Arabidopsis thaliana}	1.84	2.46	4.65	4.67	4.81
STMCD62	S-adenosylmethionine decarboxylase {Solanum tuberosum} (EC 4.1.1.50)	1.20	1.14	2.86	3.81	4.79
STMGM17	(Q9T0B2) Cellulose synthase catalytic subunit-like protein	1.84	2.37	5.91	6.22	4.79
STMCO62	(Q8RXA4) Putative glucosyltransferase	2.28	2.94	4.74	6.46	4.75
STMIR64	(Q39569) S-adenosyl-L-methionine decarboxylase proenzyme	1.11	1.26	2.94	3.85	4.75
STMCN84	bHLH transcription factor {Arabidopsis thaliana}	2.46	3.24	3.93	5.62	4.68
STMIX62	S-adenosylmethionine decarboxylase {Solanum tuberosum} (EC 4.1.1.50)	1.07	1.18	2.99	3.28	4.57
STMCG71	(Q84P53) Gamma-aminobutyrate transaminase subunit isozyme 2 (EC 2.6.1.19)	1.84	1.81	4.00	4.41	4.54
STMCB25	(Q84P53) Gamma-aminobutyrate transaminase subunit isozyme 2 (EC 2.6.1.19)	1.72	1.93	4.36	3.98	4.49
STMCQ05	Spermidine synthase 1 (Putrescine aminopropyltransferase1) (SPDSY 1) {Datura stramonium}	1.29	1.82	3.28	3.78	4.46
STMHS25	hypothetical protein T22P22.90 - Arabidopsis thaliana	0.93	1.04	1.54	2.65	4.43
STMGO18	(Q9ZSH4) 2-oxoglutarate-dependent dioxygenase	1.61	1.79	4.36	3.84	4.35
STMCB49	(Q94LC8) Putative resistance protein	1.09	1.15	1.27	2.95	4.34
STMGT55	probable zinc finger protein 58191-56692 [imported] - Arabidopsis thaliana	1.54	1.64	3.86	3.50	4.32
STMGW87	(Q8IJI4) 10b antigen putative	1.13	1.26	2.82	3.38	4.28
STMCQ62	(Q9M4X2) Putative cytochrome P450	2.01	2.42	3.92	4.63	4.24
STMCU58	(Q41384) Protein kinase (Fragment) (EC 2.7.1.-)	1.19	2.01	3.84	4.92	4.21
STMJE20	(Q9SI74) F23N19.12	1.30	2.42	3.44	4.52	4.20
STMGF88	(Q7Y236) MAP kinase-like protein	1.26	1.00	1.39	2.64	4.19
STMGN13	(Q9M4X2) Putative cytochrome P450	1.92	2.78	4.61	5.19	4.18
STMHA39	hypothetical protein T22P22.90 - Arabidopsis thaliana	0.86	0.92	1.20	2.40	4.16

Table 3.1 (cont'd) Expression ratios (wt/*jai1*)

Clone	Description	0 h	1 h	6 h	12 h	24 h
STMJP54	(Q9FZ08) Patatin-like protein 3	1.40	2.47	3.49	4.75	4.12
STMGT69	wound-induced protein Sn-1 vacuolar membrane - pepper {Capsicum annuum}	1.46	1.33	2.49	3.95	4.11
STMHI53	protein T22C5.3 [imported] - Arabidopsis thaliana	0.92	1.20	1.56	2.82	4.11
STMGA33	UDP-N-acetylmuramoylalanine-D-glutamate ligase	1.48	1.79	2.52	5.12	4.10
STMGU10	(Q8RVV4) Viroid RNA-binding protein (Fragment)	1.75	1.92	3.63	3.48	4.10
STMJB71	(Q9SLN8) Allyl alcohol dehydrogenase	0.81	0.93	1.24	2.08	4.06
STMCG85	(Q9M4X2) Putative cytochrome P450	2.26	2.61	3.92	5.15	4.05
STMGW14	unknown protein {Arabidopsis thaliana}	1.75	2.36	4.14	4.37	4.04
STMCU63	translation elongation factor EF-G {Hordeum murinum}	2.24	1.58	3.86	5.03	4.04
STMHH47	unknown protein {Arabidopsis thaliana}	1.05	1.18	1.44	2.42	4.02
STMIS51	(Q84N00) Putative class 5 chitinase	0.78	1.22	2.28	2.95	4.01
STMGC41	hypothetical protein At2g16860 [imported] - Arabidopsis thaliana	1.59	1.52	3.27	4.91	4.01
STMIN52	S-adenosylmethionine decarboxylase {Solanum tuberosum} (EC 4.1.1.50)	1.09	1.14	2.69	3.09	3.97
STMCQ53	phenylalkylamine binding protein homolog {Arabidopsis thaliana}	1.68	1.69	3.60	3.36	3.95
STMCV75	carbonate dehydratase chloroplast - common tobacco (fragment)	2.01	0.42	1.25	2.20	3.95
STMCK43	(Q8S989) Polygalacturonase-inhibiting protein	1.21	1.31	1.80	3.20	3.93
STMGN32	(Q8RVV4) Viroid RNA-binding protein (Fragment)	1.67	1.83	3.43	3.54	3.93
STMCY85	(Q84ZW1) Ent-kaurenoic acid oxidase	1.83	2.59	3.45	3.83	3.84
STMIU23	IAA-amino acid hydrolase 3 precursor. {Arabidopsis thaliana} (EC 3.5.1.-)	1.41	4.81	3.36	5.50	3.84
STMCQ85	phenylalkylamine binding protein homolog {Arabidopsis thaliana}	1.68	1.52	3.36	3.26	3.78
STMIC96	(CAE79392) PilS sensor protein precursor (EC 2.7.3.-)	1.07	1.16	2.88	3.87	3.77
STMCX02	unknown protein {Arabidopsis thaliana}	1.12	0.83	0.89	2.01	3.73
STMDE33	unknown protein {Arabidopsis thaliana}	0.95	1.21	2.22	3.05	3.69
STMJA37	Histidine decarboxylase (HDC) (TOM92). {Lycopersicon esculentum}	1.79	3.16	1.42	2.54	3.68
STMDD14	(Q8W108) AT4g14710/dl3395c (Submergence induced protein 2A)	1.00	1.13	2.14	2.58	3.67
STMCA90	hypothetical protein F4B14.250 - Arabidopsis thaliana	1.14	1.36	3.24	2.98	3.67
STMCL64	(AAR83862) Elicitor-inducible protein EIG-J7	1.54	1.86	3.56	4.35	3.59
STMGQ77	(AAR83862) Elicitor-inducible protein EIG-J7	1.14	1.29	2.48	2.88	3.57
STMIG06	(CAB71336) Putative acid phosphatase	1.00	1.28	3.77	3.46	3.55
STMGS31	(AAR82925) Coronatine-insensitive 1	3.88	5.66	4.75	2.79	3.53
STMCI91	(CAB71336) Putative acid phosphatase	1.04	1.16	3.58	4.15	3.51

Table 3.1 (cont'd) Expression ratios (wt/*jai1*)

Clone	Description	0 h	1 h	6 h	12 h	24 h
STMDH66	hypothetical protein F4B14.210 - Arabidopsis thaliana	1.17	1.08	2.01	2.53	3.50
STMCP90	(CAB71336) Putative acid phosphatase	0.90	1.31	4.50	3.90	3.49
STMDZ30	farnesyl-pyrophosphate synthetase FPS1 - tomato {Lycopersicon esculentum} (EC 2.5.1.1)	1.40	1.95	2.98	2.59	3.47
STMGE50	(Q9ZP42) Cell wall invertase precursor	1.49	1.93	2.51	4.08	3.43
STMGB09	(Q9M4X2) Putative cytochrome P450	1.98	1.66	2.73	3.67	3.40
STMDC82	phenylalkylamine binding protein homolog {Arabidopsis thaliana}	1.60	1.50	3.03	2.92	3.38
STMCK44	carbonate dehydratase chloroplast - common tobacco (fragment)	1.66	1.12	1.29	2.52	3.34
STMDS70	(Q949B4) Acetylglutamate kinase-like protein	1.43	1.84	2.36	3.53	3.33
STMCM67	IAA-Ala hydrolase (IAR3) [imported] - Arabidopsis thaliana	1.48	5.32	4.21	4.16	3.33
STMCK41	protein BYJ15 - common tobacco (fragment) {Nicotiana tabacum}	1.20	1.75	3.16	3.45	3.33
STMEP88	Histidine decarboxylase (HDC) (TOM92). {Lycopersicon esculentum}	1.66	2.88	1.40	2.41	3.26
STMCE79	farnesyl-pyrophosphate synthetase FPS1 - tomato {Lycopersicon esculentum} (EC 2.5.1.1)	1.33	1.66	2.70	2.77	3.26
STMJJ71	(Q8S0N9) Putative IAA-Ala hydrolase	1.66	2.49	3.40	3.41	3.26
STMHA52	FCA gamma {Arabidopsis thaliana}	1.02	1.51	2.31	3.01	3.25
STMID47	(Q9LKB2) Genomic DNA chromosome 3 TAC clone:K15M2	1.11	1.72	2.35	3.83	3.20
STMEC52	hypothetical protein - Arabidopsis thaliana	1.14	2.07	3.43	3.01	3.18
STMGY33	(CAF74710) MYC transcription factor (Fragment)	1.63	2.73	2.30	3.14	3.13
STMGI12	(AAR24673) At5g62200	1.47	1.67	2.89	3.58	3.11
STMGF70	(Q8S9C0) Putative cytochrome P450	1.56	1.76	2.71	3.60	3.07
STMHK41	(AAR24708) At4g25280	1.29	2.22	3.36	3.69	3.02
STMHF35	(Q8RW90) At1g12050/F12F1_8	1.09	1.67	3.00	3.46	2.98
STMDR02	unknown protein {Arabidopsis thaliana}	1.66	3.48	1.86	2.91	2.98
STMCP16	Proteinase inhibitor type II TR8 precursor. {Lycopersicon esculentum}	1.27	1.05	1.55	3.07	2.97
STMEA40	Delta-7-sterol-C5(6)-desaturase	1.70	1.72	2.72	3.32	2.97
STMCZ90	hypothetical protein T2L5.1 - Arabidopsis thaliana	1.44	3.10	1.48	2.27	2.95
STMEL23	hypothetical protein - Arabidopsis thaliana	1.24	1.90	3.39	3.06	2.77
STMJF11	hypothetical protein F14F18.60 - Arabidopsis thaliana	1.44	2.93	2.70	3.44	2.75
STMIU02	(Q9FFF4) Acetolactate synthase-like protein	1.23	2.29	3.60	4.75	2.70
STMGQ19	(Q8VXF4) Putative metallophosphatase	1.15	1.74	3.34	3.07	2.66
STMGU61	(Q8H8B2) Hypothetical protein	1.15	2.45	4.08	3.79	2.62
STMHX08	(Q9SR86) Putative aminotransferase	1.11	2.40	3.73	3.54	2.61

Table 3.1 (cont'd) Expression ratios (wt/*jai1*)

Clone	Description	0 h	1 h	6 h	12 h	24 h
STMHZ73	hypothetical protein At2g23980 [imported] - Arabidopsis thaliana	1.04	1.24	2.91	4.58	2.57
STMGS61	(Q8RY23) AT5g18860/F17K4_110	1.75	6.15	4.58	4.88	2.51
STMIX88	Formate dehydrogenase mitochondrial precursor (NAD-dependent formate dehydrogenase)	1.07	1.73	3.92	2.93	2.51
STMGX82	(Q9AWM0) P0666G04.16 protein	1.12	3.04	2.69	2.32	2.44
STMEG50	Formate dehydrogenase mitochondrial precursor (NAD-dependent formate dehydrogenase)	1.10	1.68	4.08	3.17	2.41
STMCA77	hypothetical protein At2g16810 [imported] - Arabidopsis thaliana	0.00	9.19	0.00	0.00	2.16
STMIW03	(Q9SSX8) WIZZ	1.16	5.43	1.69	1.93	2.05
STMGN14	(O81702) Gr1-protein	1.39	3.55	4.31	4.21	2.04
STMGI38	(AAS07167) Expressed protein	1.36	2.34	3.25	3.21	1.98
STMHP33	probable alpha-glucan phosphorylase - Deinococcus radiodurans (strain R1)	0.00	3.53	1.48	1.41	1.97
STMGJ89	(Q94IQ1) Peroxidase	1.07	1.33	3.04	3.05	1.95
STMCU78	Omega-6 fatty acid desaturase chloroplast precursor. {Brassica napus}	1.05	2.47	3.20	2.01	1.64
STMCK79	Omega-6 fatty acid desaturase chloroplast precursor. {Brassica napus}	1.20	2.30	3.12	2.27	1.61
STMEI66	Omega-6 fatty acid desaturase chloroplast precursor. {Brassica napus}	1.08	2.45	3.04	2.36	1.59
STMJN30	(Q9LHN3) Emb|CAB63739.1 (AT3g18860/MCB22_3)	1.07	3.45	2.04	1.85	1.42
STMGV34	Potential phospholipid-transporting ATPase 8 (Aminophospholipid flippase 8)	1.07	0.93	4.63	1.25	1.08
STMGT19	(O49150) 5-lipoxygenase	4.46	12.77	53.81	0.00	0.00
STMGX36	(O49150) 5-lipoxygenase	3.92	11.19	0.00	0.00	0.00
STMIS95	(Q9FT23) Putative glutathione S-transferase T1	0.81	0.71	0.27	0.65	0.93
STMGM71	hypothetical protein F18B3.220 - Arabidopsis thaliana	0.66	0.72	0.60	0.31	0.70
STMJB47	hsr201 protein hypersensitivity-related - common tobacco {Nicotiana tabacum}	0.91	0.85	0.70	0.25	0.60
STMID72	Probable glutathione S-transferase (Auxin-induced protein PGNT1/PCNT110). {N tabacum}	0.81	0.77	0.40	0.27	0.57
STMIH22	late-embryogenesis protein lea5 - common tobacco {Nicotiana tabacum}	0.89	0.77	0.58	0.34	0.57
STMJK24	Probable glutathione S-transferase (Auxin-induced protein PGNT1/PCNT110). {N tabacum}	0.78	0.74	0.40	0.23	0.53
STMET22	Probable glutathione S-transferase (Auxin-induced protein PGNT35/PCNT111). {N tabacum}	0.77	0.74	0.37	0.22	0.53
STMIS65	Probable glutathione S-transferase (Pathogenesis-related protein 1). {Solanum tuberosum}	0.86	0.71	0.41	0.26	0.48
STMDB44	Ribulose bisphosphate carboxylase/oxygenase activase chloroplast precursor	1.31	1.38	0.67	0.26	0.48
STMDE79	unknown protein {Arabidopsis thaliana}	0.58	0.74	0.34	0.00	0.47
STMFB15	(Q9LM08) Pepper esterase	0.82	0.63	0.40	0.26	0.44
STMES51	glutathione-conjugate transporter AtMRP4 [imported] - Arabidopsis thaliana	0.73	0.76	0.44	0.30	0.43
STMJO05	(Q9M6E7) UDP-glucose:salicylic acid glucosyltransferase	0.96	1.13	0.41	0.23	0.43

Table 3.1 (cont'd)

Clone	Description	Expression ratios (wt/*jai1*) 0 h	1 h	6 h	12 h	24 h
STMHX28	probable glucosyl transferase [imported] - Arabidopsis thaliana	0.74	1.01	0.50	0.29	0.42
STMHX67	(Q8RU72) Glucosyltransferase NTGT3 (EC 2.4.1.-)	0.93	0.90	0.19	0.21	0.42
STMJN22	probable cinnamyl-alcohol dehydrogenase - tomato {Lycopersicon esculentum}	0.87	1.12	0.55	0.30	0.41
STMEQ26	probable glucosyltransferase twi1 - tomato (fragment) {Lycopersicon esculentum}	0.97	1.10	0.46	0.33	0.39
STMJO33	unknown {Arabidopsis thaliana}	0.80	0.75	0.37	0.30	0.37
STMCG27	Ribulose bisphosphate carboxylase small chain 2A chloroplast precursor	1.08	1.10	0.61	0.38	0.35
STMJD46	(Q9LVB8) HSR203J protein-like protein	0.78	0.75	0.35	0.23	0.35
STMIO94	Glucan endo-1 3-beta-glucosidase basic isoform 2 precursor	0.86	0.83	0.77	0.40	0.34
STMDF40	Epidermis-specific secreted glycoprotein EP1 precursor (52/54-kDamedium protein)	0.73	0.57	0.35	0.29	0.34
STMIC47	CRS2-like protein - Arabidopsis thaliana	0.95	0.90	0.83	0.48	0.33
STMIU17	(Q9XF82) Fructose-1,6-bisphosphatase (EC 3.1.3.11)	0.95	0.95	0.55	1.50	0.33
STMCN81	(Q9M6E7) UDP-glucose:salicylic acid glucosyltransferase	0.90	0.92	0.33	0.18	0.32
STMEH13	Glucan endo-1 3-beta-glucosidase basic isoform 2 precursor	0.95	0.80	0.79	0.47	0.32
STMEB78	Glucan endo-1 3-beta-glucosidase basic isoform 2 precursor	0.90	0.80	0.78	0.45	0.32
STMIB46	(Q9LN73) T12C24.27	0.90	0.93	0.54	0.00	0.31
STMCY88	Chlorophyll A-B binding protein 1B chloroplast precursor (LHCII typeI CAB-1B) (LHCP)	0.94	0.90	0.45	0.00	0.29
STMIQ60	unknown protein {Arabidopsis thaliana}	0.87	0.90	0.49	0.94	0.28
STMCJ03	Chlorophyll A-B binding protein 3C chloroplast precursor (LHCII typeI CAB-3C) (LHCP)	0.96	0.83	0.50	0.78	0.27
STMJM33	(Q8H4K1) OJ1351_C05.22 protein	0.69	0.69	0.27	0.19	0.25
STMCJ04	(Q41421) Chlorophyll a/b binding protein	1.00	0.81	0.39	0.97	0.25
STMIB45	(Q8H4K1) OJ1351_C05.22 protein	0.71	0.70	0.27	0.18	0.23
STMCK28	(Q84QE4) Putative chloroplast thiazole biosynthetic protein	0.92	0.78	0.31	0.62	0.22
STMIJ42	proline-rich protein - Arabidopsis thaliana	0.67	0.59	0.25	0.18	0.22
STMCZ53	(Q84QE4) Putative chloroplast thiazole biosynthetic protein	0.92	0.73	0.28	0.47	0.19

Discussion

Our approach for determining the function of *LeAOS3* in tomato was focused on understanding the regulation of *LeAOS3* expression. Since other plant *AOS*s, particularly those involved in JA production, are known to be regulated by wounding and JA (Schaller et al., 2005), we decided to test the hypothesis that *LeAOS3* is also regulated by the JA signaling pathway. Analysis of mRNA, protein, and activity levels showed that *LeAOS3* is highly induced in roots in response to wounding and JA treatment (Figure 3.2 & 3.3). In the *jai1* mutant that lacks a functional JA-signaling pathway, the expression of *LeAOS3* in roots was completely abolished. To our knowledge, *LeAOS3* is the first example of a gene whose expression in roots is dependent on JA-signaling.

Because most of the ESTs for *LeAOS3* come from a germinating seedling cDNA library, we also analyzed expression at this stage of development. To our surprise, the level of *LeAOS3* expression in *jai1* seedlings was almost identical to wild-type (Figure 3.4). Further analysis showed that the COI1-independent expression of *LeAOS3* in germinating seedlings occurs in hypocotyl tissue rather than in root (Figure 3.7). There are likely different regulatory elements in the promoter of *LeAOS3* that are responsible for differential expression of the gene in root and hypocotyl tissue. This type of regulation has been shown for wound-induction of the *FAD7* gene in *Arabidopsis*, where different promoter elements are active in different tissues (Nishiuchi et al., 1999). If *LeAOS3* is involved in defense against soil-borne pests, constitutive expression of *LeAOS3* in hypocotyls during germination may play a role in survival prior to emergence from the soil. In contrast, the JA-inducible expression of *LeAOS3* in roots would allow

for expression of this defense under stress conditions. It is not known whether other JA-regulated root-expressed genes are constitutively expressed in hypocotyls, but genes identified in our microarray analysis are candidates to test this hypothesis. A similar expression pattern for a rice PR-protein was reported where expression in roots was inducible by JA, while expression in shoots was unaffected by JA-treatment (Hashimoto et al., 2004).

Previous studies of the 9-LOX pathway have been focused mainly on 9-LOX rather than CYP74 P450s that metabolize 9-hydroperoxy fatty acids. Several studies have shown that 9-hydroperoxides accumulate to high levels upon induction of the hypersensitive response (HR) by pathogens or elicitors (Rusterucci et al., 1999; Jalloul et al., 2002). However these studies were conducted with leaf tissue where *LeAOS3* expression has not been detected. Another study showed that in *Arabidopsis*, induction of HR coincides with the preferential accumulation of 13-hydroperoxides over 9-hydroperoxides (Montillet et al., 2002). The most compelling argument for the 9-AOS pathway not having a role in HR is the fact that *Arabidopsis* does not even have a 9-AOS, yet can exhibit a HR. Therefore it seems that the involvement of the oxylipin pathway in HR is at the level of hydroperoxides or possibly a branch other than the 9-AOS pathway. Another study in tobacco showed that suppression of a 9-LOX gene resulted in conversion of an incompatible interaction with *Phytophthora parasitica* to a compatible interaction (Rance et al., 1998). This strongly indicated a role for the 9-LOX pathway in defense against this fungal pathogen. Based on a search of the EST database at TIGR, a sequence with high similarity to LeAOS3 is present in *Nicotiana tabacum* suggesting a 9-AOS pathway could be involved in the suppression of the incompatible phenotype in

antisense 9-LOX tobacco plants. Consistent with a possible role of *LeAOS3* in defense, microarray analysis showed several genes co-regulated with *LeAOS3* that have known roles in defense.

A role for 9-AOS in plant development cannot be ruled out. Suppression of 9-LOX genes in potato resulted in fewer and deformed tubers, suggesting a role for 9-LOX pathway metabolites in tuber development (Kolomiets et al., 2001). The presence of 9-AOS activity in potato stolons (Hamberg, 2000) is consistent with this, but it is unclear whether the 9-AOS pathway is involved in tuber development. Also, no obvious developmental phenotypes have been observed for tomato *jai1* roots that lack *LeAOS3* expression. Another hypothesis is that LeAOS3 functions as a scavenger under stress conditions to prevent build-up of highly reactive hydroperoxy fatty acids. Measurements of fatty acid hydroperoxide levels in plants lacking LeAOS3 are needed to test this.

Based on the work described in this chapter, future work will be aimed at testing hypotheses for roles of the 9-AOS pathway in development and defense Using the *jai1* mutant that lacks *LeAOS3* expression in roots as a starting point is possible, but with all JA-regulated pathways affected in this mutant, it will be difficult to connect a particular phenotype to lack of 9-AOS activity. Genetic manipulation of *LeAOS3* expression in transgenic plants and testing of individual pathogens or root-feeding insects is the next step to determine what role, if any, *LeAOS3* has in defense against soil-borne invaders such as nematodes or fungal pathogens.

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Chapter 4

Biochemical Analysis of a Tomato Acyl-CoA Oxidase Required for Wound-Induced Jasmonic Acid Biosynthesis

Part of the work presented in this chapter has been published:

Li C, Schilmiller AL, Liu G, Lee GI, Jayanty S, Sageman C, Vrebalov J, Giovannoni JJ, Yagi K, Kobayashi Y, Howe GA (2005) Plant Cell 17: 971-986.

Abstract

Jasmonic acid (JA) is a lipid-derived signal that regulates plant defense responses to biotic stress. Previous work identified a mutant of tomato (*Lycopersicon esculentum*), JL1 (since renamed *acx1*), that lacks local and systemic expression of defensive proteinase inhibitors (PIs) in response to wounding (Lightner et al., 1993). Map-based cloning studies demonstrated that this phenotype results from loss of function of an acyl-CoA oxidase (ACX1A) that catalyzes the first step in the peroxisomal β-oxidation stage of JA biosynthesis (Li et al., 2005). Recombinant ACX1A exhibited a preference for C12 and C14 straight-chain acyl-CoAs and also was active in the metabolism of C18 cyclopentanoid-CoA precursors of JA. Recombinant ACX1A^{T138I}, harboring the mutation found in *acx1*, failed to bind the FAD cofactor required for catalysis, and thus showed no activity with C14 acyl-CoAs. While the overall growth, development, and reproduction of *acx1* plants were similar to wild-type plants, *acx1* plants were slightly more resistant to 2,4-DB treatment compared to wild-type. The expression of ACX1A was detected throughout the plant with the highest levels in germinating seedlings and flowers. Based on the ability of ACX1A to utilize cyclopentanoid-CoAs as substrate and the finding that *acx1* is compromised in JA biosynthesis (Li et al., 2005), we conclude that ACX1A is essential for the β-oxidation stage of JA biosynthesis.

Introduction

Jasmonic acid (JA) and its cyclic precursors and derivatives, collectively referred to as jasmonates (JAs), constitute a family of bioactive oxylipins that regulate plant responses to environmental and developmental cues. JAs are perhaps best known for their role in orchestrating plant defense responses to herbivores and certain microbial pathogens (Liechti and Farmer, 2002; Turner et al., 2002). Activation of jasmonate-mediated defenses is typically preceded by accumulation of JAs in response to biotic stress (Wasternack and Hause, 2002). JAs are synthesized by the so-called octadecanoid pathway that involves enzymes located in two different subcellular compartments (Vick and Zimmerman, 1984; Schaller, 2001; Wasternack and Hause, 2002). The first part of the pathway directs the conversion of linolenic acid to 12-oxo-phytodienoic acid (OPDA) by the sequential action of the plastid enzymes lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC). The second part of the pathway takes place in peroxisomes, where OPDA is reduced by OPDA reductase (OPR3) to give 3-oxo-2(2′[Z]-pentenyl)-cyclopentane-1-octanoic acid (OPC8). Removal of six carbons from the octanoate side chain of OPC8 yields JA.

Studies on β-oxidation in plants have mainly focused on its role in the breakdown of storage lipids in germinating seeds (Graham and Eastmond, 2002). Increasing evidence, however, indicates that nonfatty plant tissues also depend on β-oxidation for several other processes, including the synthesis of indole-acetic acid and JA (Graham and Eastmond, 2002; Zolman and Bartel, 2004). Metabolic labeling experiments with ^{18}O-OPDA provided the first evidence that the conversion of OPC8 to JA involves three

cycles of β-oxidation (Vick and Zimmerman, 1984). These early studies are supported by recent work showing that OPR3 is located in peroxisomes (Stintzi and Browse, 2000; Strassner et al., 2002), which are thought to be the exclusive site of fatty acid β-oxidation in higher plants (Graham and Eastmond, 2002; Tilton et al., 2004). Thus, it is generally assumed that the final steps of JA synthesis are catalyzed by the three core enzymes of the β-oxidation cycle, namely acyl-CoA oxidase (ACX), the multifunctional protein (MFP; containing 2-trans-enoyl-CoA hydratase and L-3-hydroxyacyl-CoA dehydrogenase activities), and 3-ketoacyl-CoA thiolase (KAT). An additional thioesterase activity is also presumably involved in the release of JA from JA-CoA, the product of the final round of β-oxidation (Figure 4.1). Identification of specific gene products that participate in the conversion of OPC8 to JA has been hampered by the fact that ACX, multifunctional protein, KAT, and thioesterases are encoded by small gene families (Graham and Eastmond, 2002; Shockey et al., 2003; Reumann et al., 2004; Tilton et al., 2004). Furthermore, isoforms within a particular enzyme family often exhibit overlapping substrate specificity (Graham and Eastmond, 2002). Recent antisense experiments conducted in *Arabidopsis thaliana* have provided evidence that specific isoforms of ACX (ACX1) and KAT (KAT2) play a role in wound-induced JA synthesis (Castillo et al., 2004). However, the precise contribution of these family members to JA production in healthy and damaged tissues remains to be determined.

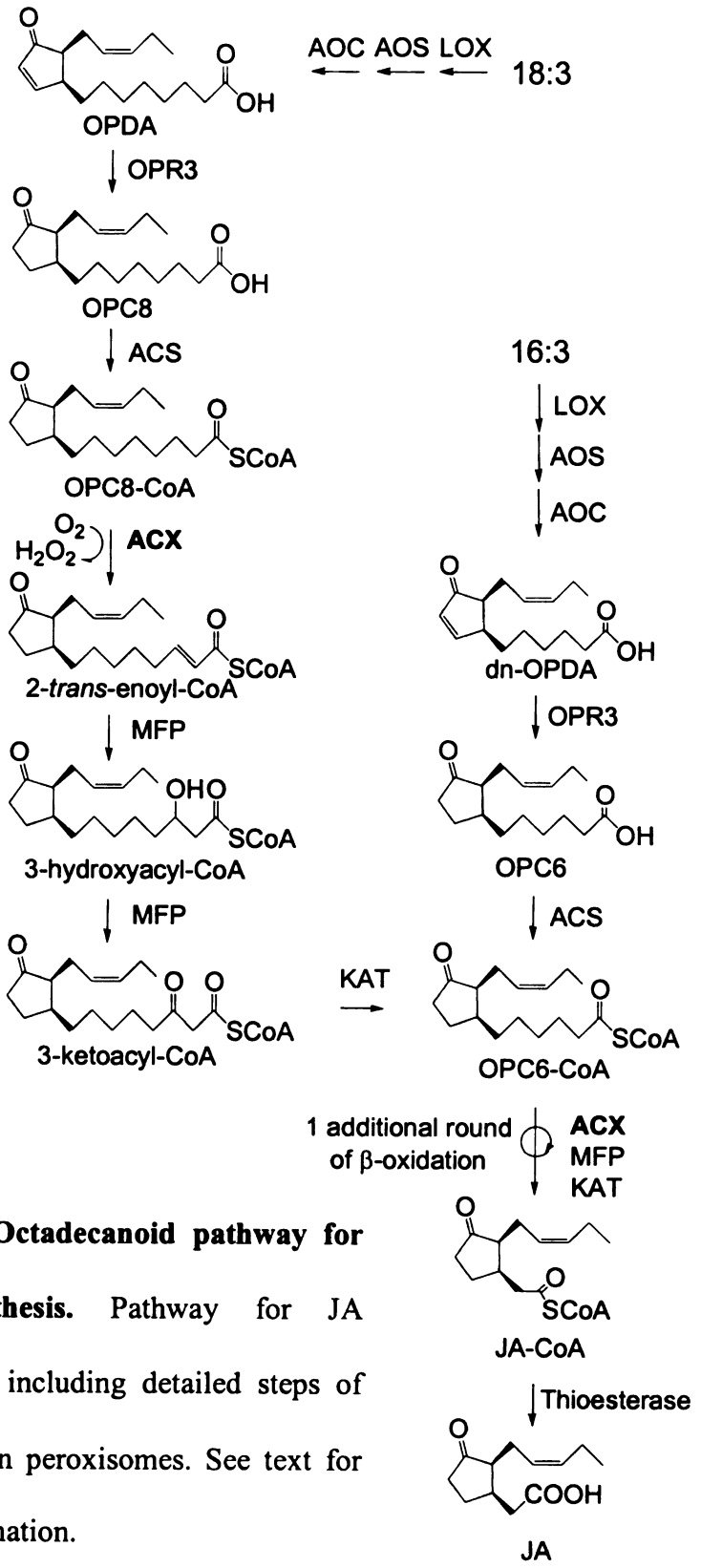

Figure 4.1 Octadecanoid pathway for JA biosynthesis. Pathway for JA biosynthesis, including detailed steps of β-oxidation in peroxisomes. See text for further information.

Here, we describe the characterization of a wound-response mutant of tomato that is deficient in JA biosynthesis. Positional cloning studies demonstrated that this defect results from loss of function of a member (ACX1A) of the ACX family of enzymes that participate in peroxisomal β-oxidation (Li et al., 2005). Results obtained from the biochemical characterization of ACX1A show its specificity profile for straight chain acyl-CoA substrates is similar to other so-called medium-long chain ACXs. We also show that ACX1A uses OPDA-CoA and OPC8-CoA as substrates. Taken together with the finding that the *acx1* mutant is compromised in JA production (Li et al., 2005), we propose that ACX1A is the major isoform essential for the β-oxidation stage of JA biosynthesis.

Materials and Methods

Plant materials and growth conditions

Tomato (*Lycopersicon esculentum*) cv Castlemart was used as the wild-type parent for all experiments. Seed for the *acx1* mutant was collected from an *acx1/acx1* homozygous line derived from successive backcrosses of the original mutant (previously called JL1; (Lightner et al., 1993) to the wild type. Seedlings were grown in Jiffy peat pots (Hummert International, Earth City, MO) in a growth chamber maintained under 17 h of light (200 $\mu E/m^2/s$) at 28°C and 7 h of dark at 18°C. Adult *acx1* plants were morphologically indistinguishable from wild-type plants and exhibited normal fruit and seed production. The germination rate of *acx1/acx1* seeds ranged between 10 and 92%,

depending on the seed batch. We reproducibly observed that the germination rate of *acx1/acx1* seed collected from field-grown plants was much higher than that of seed obtained from potted plants grown in the greenhouse (A.L. Schilmiller, C. Li, and G.A. Howe, unpublished results).

Biochemical characterization of ACX1A

A full-length *ACX1A* EST clone (cLES14H13) obtained from Clemson University Genomics Institute was used as the template for a PCR-based approach to construct a vector for expression of ACX1A with an N-terminal His_6 tag. The forward primer (5'-GAGCTCGTAAGAGAGATGGAGGGTGTA-3') was designed with a *Sac*I site and the reverse primer (5'-CCGCTCGAGCGGAACAGTTTGCTGCAGCTC-3') spanned a *Pst*I site located in the 3'-untranslated region of *ACX1A*. PCR amplification yielded a 2047-bp product that was subcloned into pGEM-Teasy (Promega). After digestion with *Sac*I and *Pst*I, the *ACX1A* cDNA insert in this construct was cloned into the same sites of the expression vector pQE30 (Qiagen, Valencia, CA). The resulting construct, which added 19 amino acids (MRGSHHHHHHGSACELVRE) to the N-terminus of ACX1A, was transformed into the Rosetta strain of *E. coli* (Novagen, Madison, WI). The expression construct for ACX^{T138I} was prepared by the same procedure, except the template for PCR was the *ACX1A* cDNA derived from the *acx1* mutant. Expression of recombinant ACX1A proteins ($ACX1^{wt}$ and $ACX1^{T138I}$) was initiated by inoculating 1 mL of an overnight culture into 200 mL of Terrific Broth medium supplemented with 100 μg/mL of ampicillin and 12.5 μg/mL of chloramphenicol. Bacteria were grown at 37°C in a shaker (250 rpm) to an OD_{600} of 0.5. Cultures were then cooled to 30°C, and isopropyl-

thio-β-D-galactopyranoside (Roche, Indianapolis, IN) was added to a final concentration of 0.1 mM. The induced cultures were incubated at 30°C for 18 h with gentle shaking (120 rpm). Cells were harvested by centrifugation and either stored at -20°C or used immediately for ACX1 purification. The cell paste was resuspended in 50 mM potassium phosphate, pH 7.6, 150 mM NaCl, and 10% (v/v) glycerol and then lysed by sonication. His-tagged ACX1 proteins were purified from the cleared lysate by nickel affinity chromatography (nickel-nitrilotriacetic acid agarose resin; Qiagen) according to manufacturer's directions. During the purification procedure, FAD (Sigma-Aldrich, St. Louis, MO) was added to all buffers at a final concentration of 10 μM. Protein measurements were performed using the BCA assay (Pierce, Rockford, IL), with BSA as a standard.

ACX activity with commercially available acyl-CoA substrates (Sigma-Aldrich) was measured with an H_2O_2-coupled spectrophotometric assay as described previously (Hyrb and Hogg, 1979). Typical enzyme assays contained 0.5 μg of affinity-purified ACX1 and 50 μM of acyl-CoA substrate. ACX1A-catalyzed metabolism of OPDA-CoA and OPC8-CoA was measured with an enzymatic colorimetric assay (Roche) that is typically used to measure free fatty acid levels in serum or plasma (Shimizu et al., 1980). The method is based on the activation of nonesterified fatty acids by ACS to the corresponding CoA ester, followed by detection of ACX catalyzed H_2O_2 production (Shimizu et al., 1980). The test kit provided by the manufacturer uses a yeast ACS and an unspecified ACX for the coupled reaction. For our studies, the ACX provided by the manufacturer was replaced with affinity-purified ACX1A. Fatty acid substrate (dissolved in 0.25% Triton X-100) was added to 100 μL of the manufacturer's reaction mix A

(containing ATP, CoA, ACS, and sodium phosphate, pH 7.8). Concentrated stocks of OPDA and OPC8 in ethanol were diluted directly in 0.25% Triton X-100 (v/v). The sodium salt of myristic acid (14:0) was prepared according to Shimizu et al. (1980). Reactions were allowed to proceed for 30 min at 25°C, at which time excess CoA was trapped by alkylation via the addition of 5 μL of N-ethyl-maleinimide. The ACX reaction was then initiated by addition of 1 μg of purified ACX1A. H_2O_2 produced in this reaction was used by a peroxidase to convert 2,4,6-tribromo-3-hydroxy-benzoic acid and 4-aminoantipyrine to a red dye that was measured at 546 nm with a Beckman DU530 spectrophotometer (Fullerton, CA). OPC8 was chemically synthesized according to the procedure of Ainai et al. (2003). NMR spectroscopy showed that the OPC8 was >97% pure and that the ratio of the cis and trans isomer was >20:1. OPDA was obtained from Larodan Fine Chemicals (Malmo, Sweden).

Gene expression analysis

RNA extraction was performed as previously described (Howe et al., 2000). cDNA clones for *ACX1A* (cLES14H13) and *eIF4A* (cLED1D24) were used to prepare probes for hybridization. Duplicate gels were stained with ethidium bromide to assess the quality of the RNA.

Antibody preparation and protein blot analysis

Recombinant His-tagged ACX1A was purified from *E. coli* as described above and sent for polyclonal antibody production (Cocalico Biologicals, Reamstown, PA) in rabbits following their standard protocol for inoculation. Total protein was extracted from

various tissues by grinding in liquid nitrogen and adding extraction buffer (50 mM sodium phosphate pH 7.0, 150 mM NaCl, 10% glycerol, and Roche mini-complete protease inhibitors) at a buffer to tissue ratio of approximately 2:1. Plant debris was removed by centrifugation at 13,000 x g and protein concentration was measured in the supernatant using the BCA assay (Pierce, Rockford, IL) with BSA as a standard. Protein was separated on 10% SDS-polyacrylamide gels and transferred to Imobilon-P membranes (Millipore, Bedford, MA) using standard procedures (Harlowe and Lane, 1988). Membranes were probed with anti-ACX1A antibodies at a 1:1000 dilution in Tris-buffered saline (TBS) containing 1% nonfat milk (Krogers) and 0.1% Tween 20 (Sigma). Antigen-antibody complexes were probed using a horseradish peroxidase conjugated secondary antibody (Sigma) at 1:25,000 dilution in TBS + 1% nonfat milk and 0.1% Tween 20 followed by detection using the Supersignal West Pico Chemiluminescent substrate (Pierce, Rockford, IL).

2,4-DB treatment of germinating seedlings

Seeds of wild-type (Castlemart) and *acx1* were surface sterilized with 40% beach and rinsed several times with sterile water, then germinated for two days on plates containing MS media (Plant Media, Dublin, OH). After three days, plants were transferred to fresh MS plates containing various concentrations of 2,4-dichlorophenoxy butyric acid (Sigma). Root lengths were measured five days after transfer.

Results

The wound-response phenotype of acx1 results from a defect in ACX

Genetic mapping of *acx1* narrowed the location to a single *Lycopersicon cheesmanii* BAC clone, which was sequenced (Li et al., 2005). Two candidate genes located adjacent to each other were identified as putative ACX-encoding genes, which we designated *LcACX1A* and *LcACX1B* (Figure 4.2). Given the likely role of ACX in JA biosynthesis and wound signaling (Vick and Zimmerman, 1984; Castillo et al., 2004), subsequent experiments were focused on testing the hypothesis that the wound-response phenotype of *acx1* results from a defect in *ACX1A* or *ACX1B*.

Plant ACXs comprise a family of flavoenzymes that catalyze the initial step of peroxisomal β-oxidation of a variety of acyl-CoA substrates (Graham and Eastmond, 2002). As a first step toward characterizing the tomato *ACX1A* and *ACX1B* genes, we used RT-PCR to obtain full-length cDNAs (designated *LeACX1A* and *LeACX1B*) from wild-type (cv Castlemart) *L. esculentum*. The predicted amino acid sequence of the two tomato ACXs are 81.7% identical. ACX1A and ACX1B are most similar to ACXs characterized from *Arabidopsis* (80.1 and 73.3% identical to AtACX1, respectively) and soybean (81.8 and 75.7% identical to GmACX1-1, respectively) that have broad specificity for medium- to long-chain acyl-CoAs (Figure 4.3)(Hooks et al., 1999; Agarwal et al., 2001). Both LeACX1A and LeACX1B possess a C-terminal peroxisomal targeting signal type 1 (PTS1) motif (Figure 4.2) (Reumann et al., 2004). The C-terminal region of ACX1B lacks a 15–amino acid sequence that is highly conserved in other medium- to long-chain plant ACXs (Figure 4.2). Comparison of the ACX1B genomic

Figure 4.2 Sequence alignment of LeACX1A and LeACX1B. Predicted amino acid sequences for ACX1A and ACX1B were aligned using the Multalin sequence analysis package (http://prodes.toulouse.inra.fr/multalin/multalin.html)(Corpet, 1988). The "+" denotes the Thr residue (Thr138) in LeACX1A that is affected by the *acx1* missense mutation. Asterisks (*) denote residues that are missing in the deduced sequence of LeACX1B.

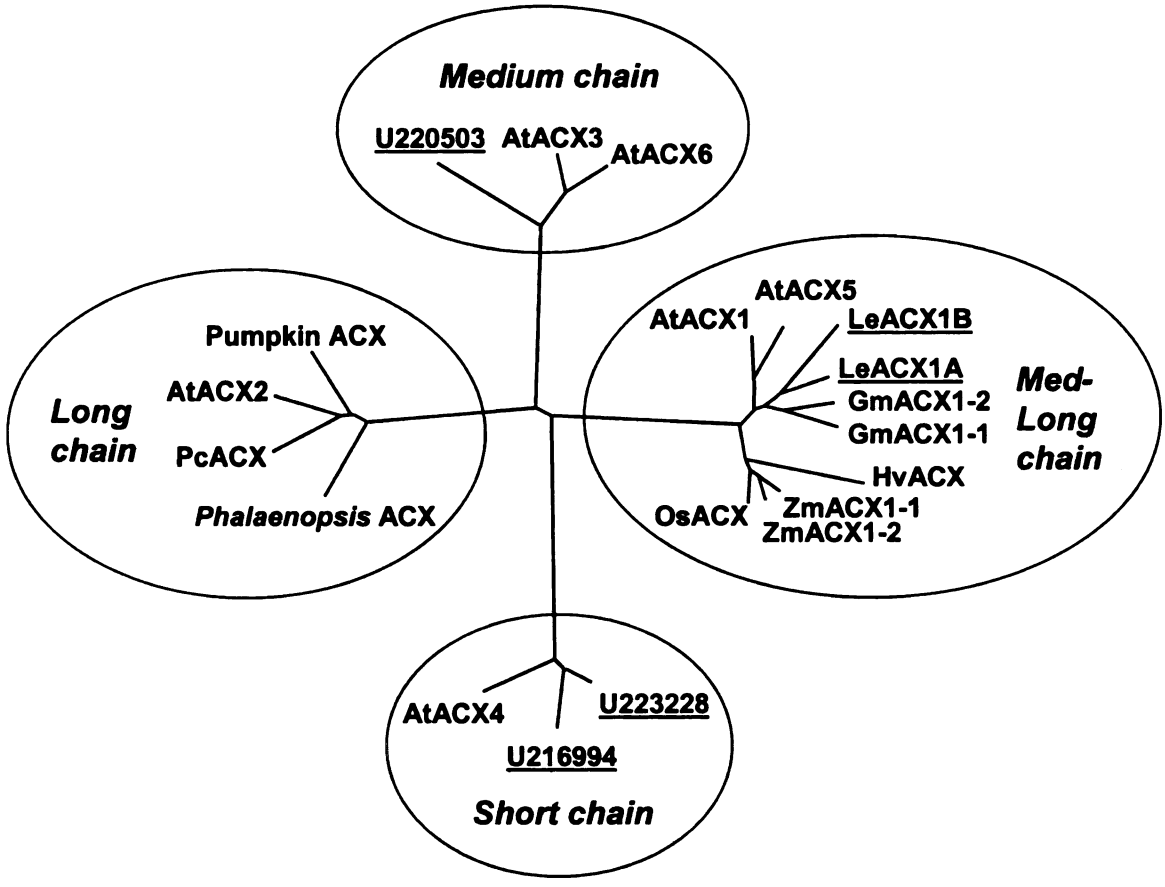

Figure 4.3 Plant ACX phylogeny. An unrooted phylogenetic tree is shown, constructed from deduced amino acid sequences from various plant *ACX*s. Gray boxes indicate the substrate preference of members of each circled group for acyl-CoA substrates of various chain lengths. Tomato sequences are underlined. U220503, U216994, and U223228 are predicted tomato ACX sequences compiled from EST sequence data at the Solanaceous Genomics Network. (http://www.sgn.cornell.edu)

and cDNA sequences suggested that this polymorphism results from alternative splicing at cryptic donor and acceptor splice sites.

Comparison of the sequence of wild-type- and *acx1*-derived *ACX* cDNAs revealed a single C-to-T nucleotide substitution in *LeACX1A*. No sequence differences were found in LeACX1B. The single-base change in *LeACX1A*, which was confirmed by sequencing of PCR-amplified genomic DNA from *acx1*, is predicted to replace a highly conserved (i.e., invariant among all plant and animal ACXs) Thr residue at position 138 with an Ile. The three-dimensional structure of mammalian ACX (Nakajima et al., 2002) and its conserved mitochondrial counterpart, acyl-CoA dehydrogenase (Kim and Miura, 2004), has shown that this Thr binds the flavin ring of FAD that defines the active site of the enzyme.

Genetic complementation experiments were performed to determine whether the missense mutation in *LeACX1A* was responsible for the deficiency in wound-induced PI-II expression in *acx1* plants. The wild-type *LeACX1A* cDNA was placed under the control of the 35S promoter of *Cauliflower mosaic virus* in T-DNA vector pBI121. This construct was introduced into the *acx1* genetic background by *Agrobacterium tumefaciens*–mediated transformation. From 33 independent *35S-ACX1A* transgenic plants confirmed by genomic PCR, 20 primary lines (T_0) showed normal levels of wound-induced PI-II expression both in the wounded leaf and in the systemic unwounded leaves (Figure 4.4). Further characterization of two of these lines showed that the complemented phenotype was inherited in the T_1 generation (data not shown). These results demonstrate that the wound-response phenotype of *acx1* results from loss of function of ACX1A.

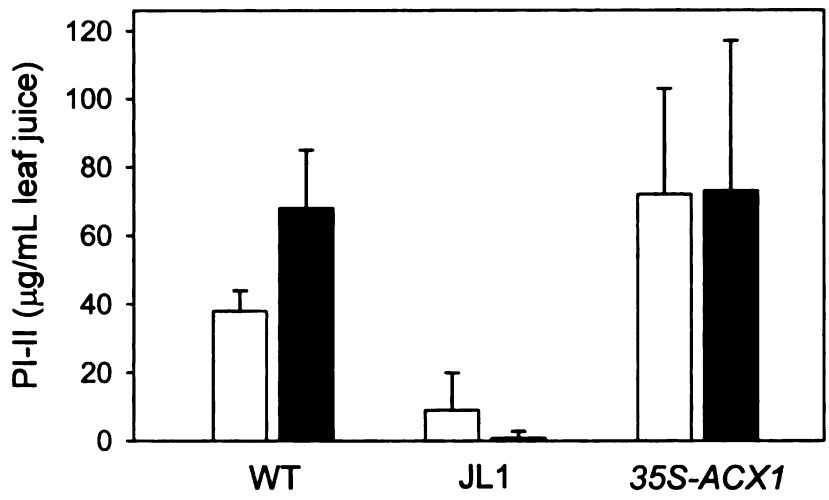

Figure 4.4 Genetic complementation of the wound-response phenotype of *acx1*. Wild-type, *acx1*, and *35S-ACX1A*-expressing mutant plants (at the five- to six-leaf stage) were wounded once across the midvein of each leaflet on the two lower leaves. The same leaflets were wounded again 3 hrs later. Twenty-four hours after the initial wounding, PI-II levels in the lower wounded leaves (open bars) and the upper unwounded leaves (closed bars) were measured. Data show the mean ± SD (n = 7 for the wild type and *acx1*; n = 20 independent transgenic lines confirmed by PCR to contain the *35S-ACX1A* transgene). PI-II levels in unwounded control plants were below the detection limit of the assay (~5 µg PI-II/ml leaf juice).

To determine whether this mutation (T138I) affects the biochemical activity of ACX1A, we expressed His-tagged derivatives of the wild-type ($ACX1^{wt}$) and mutant ($ACX1^{T138I}$) proteins in *Escherichia coli*. Affinity-purified $ACX1^{wt}$ displayed 381- and 452-nm absorption peaks that are indicative of flavoenzymes (Figure 4.5A). An equivalent amount of affinity-purified $ACX1^{T138I}$ lacked this spectral signature. In vitro activity assays with straight-chain acyl-CoAs showed that recombinant $ACX1^{wt}$ has a preference for C12 and C14 substrates (Figure 4.5B). The enzyme was less active with C18 and C20 substrates and showed little or no activity with short-chain (≤C6) acyl-CoAs. $ACX1^{T138I}$ showed no detectable activity against 14:0-CoA, which was a preferred substrate of the wild-type enzyme (Figure 4.6). These results indicate that the T138I mutation in *acx1* renders ACX1A inactive, most likely by disrupting FAD binding to the apoprotein.

Figure 4.5 UV/vis spectra of purified recombinant ACX1A and ACX1A^{T138I} and substrate specificity of ACX1A.

(A) UV-visible spectra of affinity-purified recombinant ACX1A (solid line) and ACX1A^{T138I} (dotted line) were recorded at a protein concentration of 2 mg/ml. Absorption peaks at 381 nm and 452 nm are indicative of FAD binding.

(B) Substrate specificity of ACX1A. The activity of recombinant ACX1A against various fatty acyl-CoA esters was measured using a spectrophotometric assay linked to H_2O_2 production. Data show the mean ± SD from three replicate assays with the same extract, and are representative of experiments performed with independent enzyme preparations.

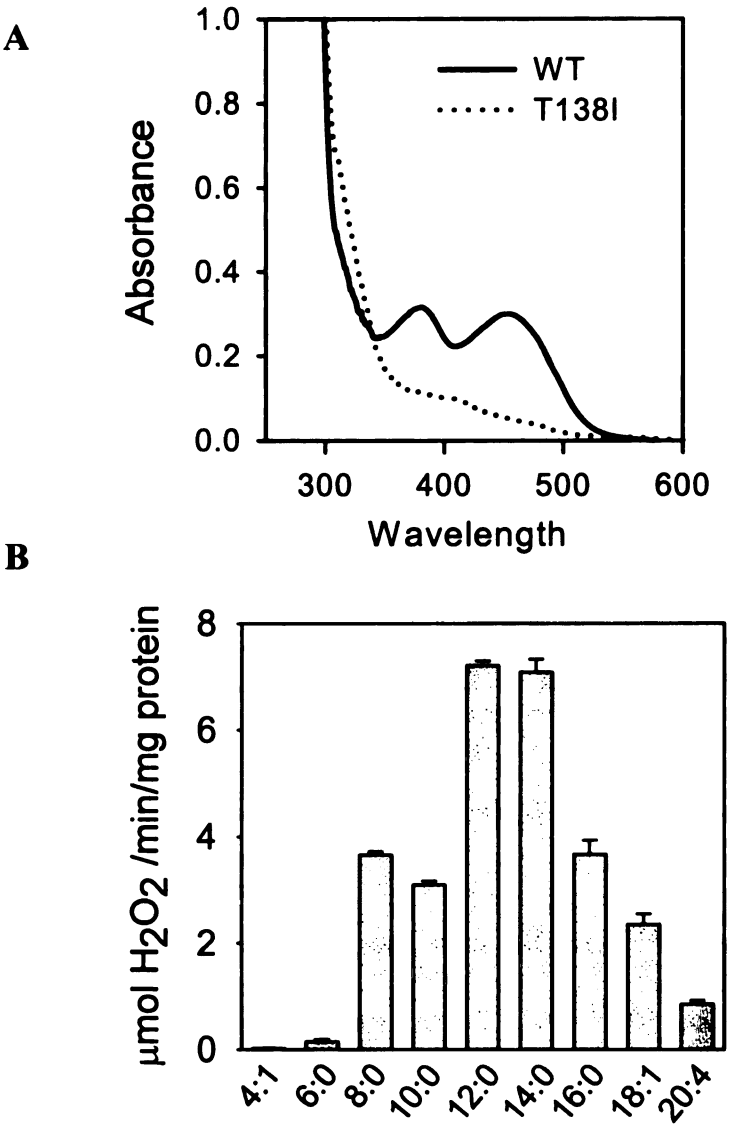

Figure 4.5 UV/vis spectra of purified recombinant ACX1A and ACX1A^{T138I} and substrate specificity of ACX1A.

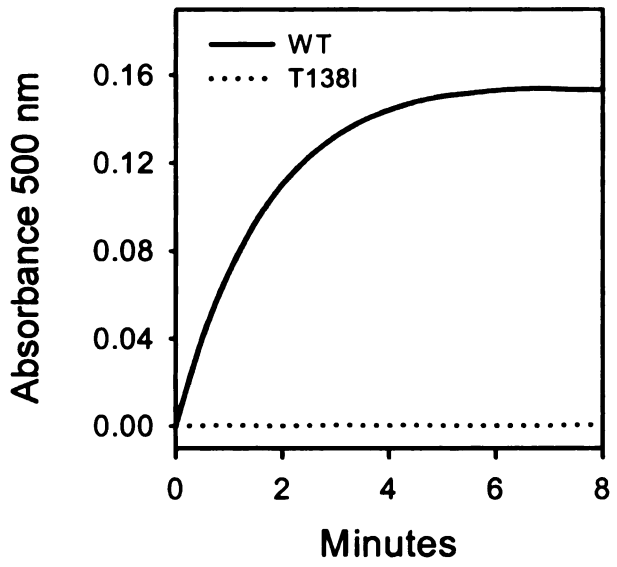

Figure 4.6 ACX activity assay with ACX1A^{T138I}. ACX activity was measured for ACX1A (solid line) and ACX1A^{T138I} (dotted line) against 14:0-CoA. The change in absorbance at 500 nm results from production of a colored product from a peroxidase catalyzed reaction utilizing the H_2O_2 released from the ACX reaction.

ACX1A is required for JA biosynthesis

Current knowledge of the JA biosynthetic pathway predicts that OPC8-CoA is the substrate for entry into the β-oxidation cycle via the action of ACX (Figure 4.1). To determine whether ACX1A is capable of metabolizing OPC8-CoA, we used an in vitro assay that couples ACX1A to a yeast acyl-CoA synthetase (ACS) that activates the fatty acid substrate to the corresponding CoA ester. Addition of 50 μM OPC8 to the assay resulted in ACX1A activity that was significantly greater than that obtained with the same concentration of 14:0 (Figure 4.7). Control experiments showed that ACX1A activity in these experiments was dependent on ACS and CoA and was proportional to the amount of substrate added (data not shown). Interestingly, OPDA was comparable to OPC8 in its ability to promote ACX1A activity. These results provide evidence that ACX1A can metabolize OPC8-CoA and that reduction of the double bond in the cyclopentenone ring of OPDA is not required for the enzyme's activity.

Developmental expression of ACX1A in tomato

RNA- and western-blot analyses were used to investigate the abundance of *ACX1A* mRNA and its corresponding protein in various tissues of wild-type and *acx1* tomato plants. *ACX1A* transcripts were detected in all tissues examined with highest expression seen in germinating seedlings and flowers (Figure 4.8). Though *acx1* contains a point mutation in *ACX1A*, expression in the mutant is unchanged compared to wild-type. Using polyclonal antibodies raised against recombinant ACX1A, we also examined the levels of ACX1A protein in various tissues (Figure 4.9). The antibody cross-reacted with a protein at the expected size of 74 kD. In wild-type, ACX1A protein was

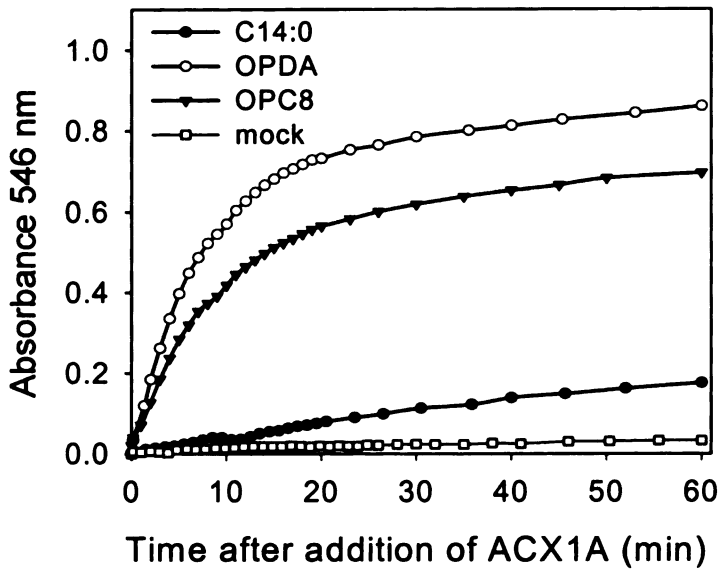

Figure 4.7 ACX1A activity against OPDA and OPC-8 in a coupled assay with acyl-CoA synthetase. ACX1A activity against OPDA-CoA and OPC8-CoA was tested using the corresponding free acids in a coupled reaction using an ACS with recombinant ACX1A. The indicated substrates (5 μL in 0.25% Triton X-100, 50 μM final concentration) or mock control were added to a reaction mixture containing a yeast ACS, CoA, and ATP. After 0.5 hrs at 25°C, the reaction was terminated by addition of N-ethylmaleinimide and the ACX reaction was initiated by addition of 1 μg of purified ACX1A. ACX1A activity was monitored spectrophotometrically via the production of a colored product from a peroxidase catalyzed reaction utilizing the H_2O_2 released from the ACX reaction.

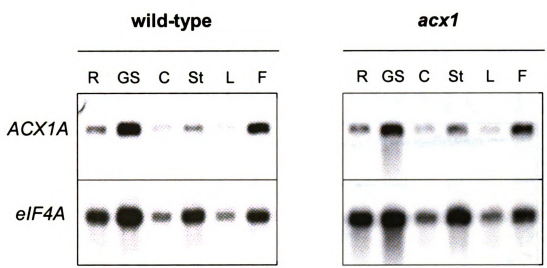

Figure 4.8 *ACX1A* expression in various tissues. RNA was extracted from roots (R), germinating seedlings (GS), cotyledons (C), stems (St), leaves (L), and unopened flower buds (F) of wild-type and *acx1* plants. Identical RNA blots were probed with either *ACX1A* or *eIF4A* (loading control) cDNA probes.

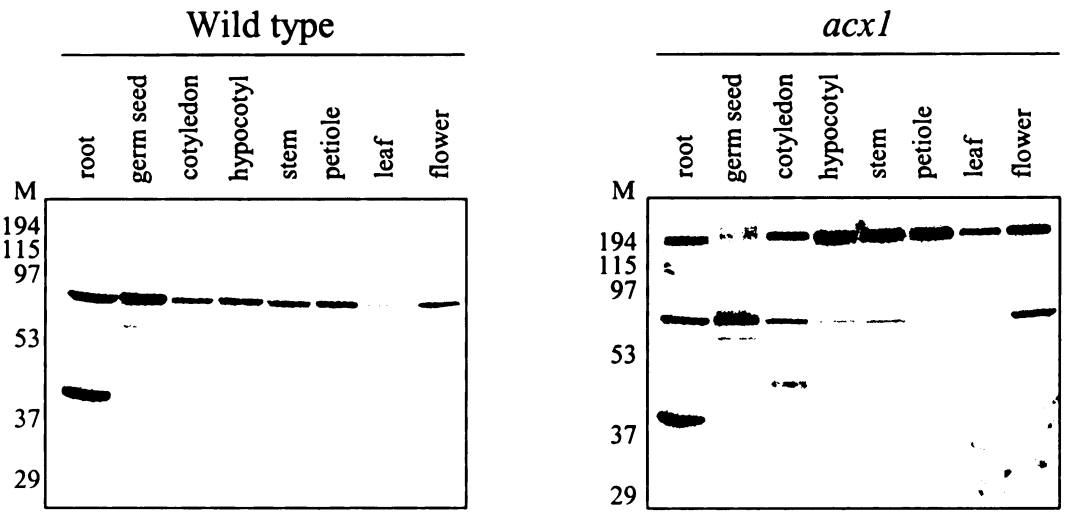

Figure 4.9 Analysis of ACX1 protein in various WT and *acx1* tissues. Twenty-microgram samples of protein from various tissues of wild type (left) or *acx1* (right) was separated by SDS-PAGE, transferred to PVDF membranes, and probed with antiserum raised against recombinant ACX1A. M, sizes of molecular weight markers.

detected in each of the tissues examined in a pattern similar to that of its transcript. When ACX1A protein was analyzed in extracts from *acx1* tissues, protein of the expected size was reduced compared to that of wild-type. However, a much larger cross-reacting band appeared in *acx1* extracts not seen in wild-type, and also not seen in the corresponding pre-immune blots (data not shown).

Involvement of ACX1A in β-oxidation of other substrates

It has been shown for all ACXs studied to date that they tend to exhibit highly variable substrate specificities, in terms of the chain length of fatty acyl-CoAs (Graham and Eastmond, 2002). This was also seen for ACX1A as shown above (Figure 4.5B). Since ACX1A was more active with OPDA-CoA and OPC8-CoA (both containing a five-carbon ring), we also tested whether ACX1A could participate in β-oxidation of another cyclic substrate, 2,4-dichlorophenoxybutryic acid (2,4-DB; containing a six-carbon ring). 2,4-DB can undergo one round of β-oxidation to be converted to the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4D), which has been shown to inhibit root growth of germinating seedlings (Hayashi et al., 1998). Root lengths were measured five days after two-day old wild-type and *acx1* germinating seedlings were transferred to plates containing media supplemented with 2,4-DB. Compared to seedlings that were untreated or grown on 0.5 μM 2,4-DB, wild-type seedlings treated with 1 μM 2,4-DB showed significant reduction in root growth ($p=0.026$, Mann-Whitney Rank Sum test)(Figure 4.10). 10 μM was approximately the saturating concentration of 2,4-DB needed to give maximum root growth inhibition for wild type. The root length for *acx1* germinating seedlings grown on 1 μM 2,4-DB was not significantly different than either

untreated wild-type, untreated *acx1*, or *acx1* grown on 0.5 μM 2,4-DB, indicating the loss of ACX1A impairs β-oxidation of 2,4-DB. This suggests that ACX1A can participate in the β-oxidation of other cyclic substrates. At 10 μM however, root growth inhibition was similar to wild type, likely indicating that another ACX isoform with a lower affinity for 2,4-DB can substitute for the loss of ACX1A at higher concentrations.

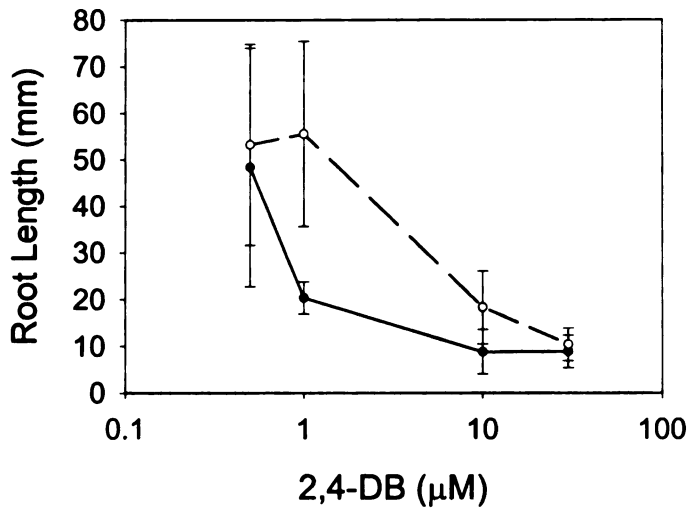

Figure 4.10 2,4-DB treatment of wild-type and *acx1* germinating seedlings. Seed of wild type and *acx1* were germinated on MS plates, and transferred after two days to plates containing various concentrations of 2,4-dichlorophenoxy butyric acid (2,4-DB). Root lengths were measured five days after transfer (wild-type, solid line; *acx1*, dashed line).

Discussion

Here we provide several lines of evidence demonstrating that the wound-signaling defect in the JL1 mutant of tomato (renamed here *acx1*) results from a block in the β-oxidation stage of the octadecanoid pathway. First, *acx1* plants harbor a defective member (ACX1A) of the ACX family of enzymes that catalyze the first step in the β-oxidation cycle. Second, recombinant ACX1A metabolizes OPC8-CoA, which is the presumptive substrate for the first β-oxidation cycle in the JA biosynthetic pathway. Finally, it has been shown that *acx1* leaves accumulate very little (~5% wild-type levels) JA in response to wounding (Li et al., 2005). We thus conclude that ACX1A is responsible for the majority of wound-induced JA in tomato leaves.

The deduced amino acid sequence and substrate specificity of LeACX1A is most similar to that of ACX1 and ACX5 of *Arabidopsis* and two ACXs from soybean (Hooks et al., 1999; Eastmond et al., 2000; Agarwal et al., 2001). These ACXs have relatively broad substrate specificity and, as yet, no definitive physiological function. Several observations lead us to propose that LeACX1A and other members of the so-called medium- to long-chain subfamily of ACXs (Figure 4.3) play a prominent role in JA biosynthesis. First and foremost is the JA-deficient phenotype of *acx1* plants. Second, results from the coupled ACS-ACX assay indicate that ACX1A exhibits a preference for C18 cyclopentanoid-CoAs over 14:0-CoA (Figure 4.7). This observation suggests that ACX1A and related enzymes possess structural features that facilitate the metabolism of C18 cyclopentanoid fatty acids. Third, recent studies have implicated *Arabidopsis* ACX1 in wound-induced JA synthesis and expression of wound responsive genes (Castillo et al.,

2004). Unlike the severe JA deficiency in tomato *acx1* plants, *Arabidopsis acx1* antisense lines retained 50 to 60% of wild-type levels of wound-induced JA. This relatively weak phenotype may reflect incomplete suppression of AtACX1 or the capacity of other ACXs (e.g., ACX5) to participate in JA biosynthesis in wounded *Arabidopsis* leaves. Hooks et al. (1999) reported that anti-ACX1 lines of *Arabidopsis* exhibit a modest reduction in root growth, but JA levels were not assessed in that study.

Identification of LeACX1A as an essential component of the octadecanoid pathway supports the original proposal by Vick and Zimmerman (1984) that JA biosynthesis involves β-oxidation enzymes that remove six carbons from the octanoate side chain of OPC8. The presence of a PTS1 sequence at the C terminus of LeACX1A, together with the fact that fatty acid β-oxidation in plants occurs in peroxisomes, are consistent with the notion that ACX1A is a peroxisomal protein. Subcellular localization experiments are needed to confirm this. Based on the ability of ACX1A to use OPC8-CoA and the location of OPR3 in peroxisomes (Strassner et al., 2002), our results indicate that the likely in vivo substrate for ACX1A is a peroxisomal pool of OPC8-CoA (Figure 4.1). At present, the temporal sequence of metabolic events involved in the conversion of plastid-localized OPDA to OPC8-CoA remains to be established. One possibility is that OPDA is transported from the plastid as a CoA ester and is then converted by OPR3 to OPC8-CoA in the peroxisome. Because OPR3 readily accepts free OPDA as a substrate (Schaller, 2001; Strassner et al., 2002), an alternative scheme is that OPR3 first reduces OPDA to OPC8, which is then converted to OPC8-CoA. Identification of enzymes involved in the synthesis and transport of C18 cyclopentanoid-CoAs may help to distinguish these possibilities.

Our results do not exclude the possibility that conversion of OPC8-CoA to JA-CoA involves multiple ACXs that specifically act on OPC8-CoA, OPC6-CoA, or OPC4-CoA. However, the ability of LeACX1A to metabolize a broad range of fatty acid chain lengths (Figure 4.5B) argues against this hypothesis. Determination of the relative specificity of ACX1A for different OPC-CoA derivatives would be helpful to further address this issue, as would the identification of OPC-CoA intermediates that accumulate in *acx1* tissues. In considering the role of ACX1A in JA biosynthesis, it is noteworthy that the enzyme accepts both OPC8- and OPDA-CoAs as substrates in the ACS-ACX coupled assay (Figure 4.7). Because we did not determine the extent to which each substrate was activated to the CoA ester after the ACS reaction, it is not possible to draw firm conclusions about the relative preference of ACX1A for different substrates. However, assuming that OPC8 and OPDA were equally converted to the CoA ester, it would appear that ACX1A does not discriminate between the cyclopentenone (i.e., OPDA) and the cyclopentenone (i.e., OPC8) derivatives. This observation raises the possibility that OPR3 activity is not strictly required for entry of OPDA into the β-oxidation cycle. Complete β-oxidation of OPDA is expected to yield 4,5-didehydro-JA. Such a pathway may explain the production of this JA derivative in fungi (Miersch et al., 1989) and plants (Dathe et al., 1989).

The *acx1* mutation affects a Thr residue (Thr138) that plays a critical role in positioning the FAD cofactor at the active site of the enzyme (Kim and Miura, 2004). However, the point mutation in *acx1* does not seem to cause a change in the steady state levels of the *acx1* transcript (Figure 4.8). Analysis of ACX1A protein levels in *acx1* suggest that $ACX1^{T138I}$ is mostly present as a multimer that is not separated even by

SDS-PAGE (Figure 4.9). It is not clear why such a high molecular weight form accumulates, but one possible cause of the aggregation is improper folding due to lack of FAD binding (Figure 4.5A). This result together with the observation that recombinant ACX1^{T138I} lacked detectable activity against 14:0-CoA indicates that *acx1* is most likely a null mutation. If this is the case, the residual JA production in *acx1* plants can be attributed to one or more other ACX family members. The sequence of BAC232L13 revealed that LcACX1A is located adjacent to a closely related family member, designated LcACX1B. Although RT-PCR experiments confirmed that ACX1B is expressed in *L. esculentum* leaves, the presence of a 15–amino acid deletion in the deduced sequence of ACX1B (Figure 4.2) raises the question of whether this gene encodes an active enzyme. Attempts to express ACX1B in *E. coli* resulted in failure to detect accumulation of the fusion protein (L. Katsir, A.L. Schilmiller, and G.A. Howe, unpublished results). Inspection of the tomato EST database (http://www.sgn.cornell.edu) provided evidence for additional ACX genes that are homologous to the short-chain (AtACX4) and medium-chain (AtACX3) ACXs of *Arabidopsis* (Figure 4.3). An additional partial-length sequence (SGN-U229242) appears to be homologous to the *Arabidopsis* long-chain ACX (AtACX2). Given the distinct but overlapping substrate specificity of different ACX family members (Eastmond et al., 2000; Graham and Eastmond, 2002), it is reasonable to propose that one or more of these tomato ACXs is responsible for the residual JA production in *acx1* leaves.

In higher plants, β-oxidation has been extensively studied for its role in converting storage lipids to acetyl-CoA during early stages of seedling development. The importance of this process in the plant life cycle is highlighted by the fact that mutants

defective in peroxisomal β-oxidation often exhibit developmental arrest at the seedling stage (Hayashi et al., 1998; Zolman et al., 2001; Footitt et al., 2002). It is possible that ACX1A, in addition to its role in JA biosynthesis, also is involved in fatty acid catabolism during seed germination and seedling growth. This idea is supported by the ability of ACX1A to use straight-chain fatty acyl-CoAs (Figure 4.5B) as well as the accumulation of *ACX1A* transcripts and its encoded protein in germinating tomato seedlings (Figure 4.8, 4.9). On the other hand, the ability of *acx1* seedlings to establish photoautotrophic growth in the absence of sucrose (data not shown) indicates that if ACX1A does participate in seed lipid mobilization, this role is not required for seedling establishment. A nonessential role for ACX1A in seed lipid mobilization is consistent with detailed biochemical analysis of the ACX family in *Arabidopsis* (Hooks et al., 1999; Eastmond et al., 2000; Eastmond et al., 2000; Froman et al., 2000). These collective studies indicate that the substrate specificities of AtACX2 and AtACX3 are likely sufficient to metabolize, in the absence of AtACX1, the full range of medium- and long-chain acyl-CoAs that exist in vivo.

β-Oxidation in nonfatty plant tissues has been implicated in diverse physiological processes, including floral development, synthesis of indole-acetic acid and JA, and production of acetyl-CoA substrate for primary and secondary metabolism (Graham and Eastmond, 2002; Zolman and Bartel, 2004). Aside from defects in JA biosynthesis and JA-mediated defense responses, adult *acx1* plants did not exhibit overt morphological or reproductive phenotypes. Based on these observations, we suggest that a primary function of ACX1A in tomato leaves is catalysis of JA synthesis. However, with the ability of ACX1A to also use 2,4-DB, demonstrated by the reduced sensitivity of *acx1* for

this proherbicide, we cannot rule out the possibility that ACX1A functions in β-oxidation of other substrates.

We previously showed that the F-box protein CORONATINE INSENSITIVE1, which is an essential component of the JA signaling pathway, is required for female fertility in tomato (Li et al., 2004). This finding implies that normal female reproductive development in tomato requires the action of endogenous JAs, which are known to accumulate to high levels in reproductive organs (Hause et al., 2000). Although *acx1* homozygotes produce normal amounts of seed, we have observed that the rate of *acx1* seed germination varies considerably (from <15 to >90%) between different seed batches. This variability may be related to environmental growth conditions (see Methods). The ability of *acx1* plants to produce viable seed is similar to that of the *spr2* tomato mutant that is defective in the production of linolenic acid, the major precursor of JAs (Li et al., 2003). We thus suggest that reproductive tissues of *acx1* plants, like those of *spr2*, are capable of producing JAs at a level that is sufficient to promote seed production. Assuming that *acx1* is a null mutation, this explanation implies that other ACX isoforms can participate in JA biosynthesis in reproductive tissues.

In conclusion, we show that ACX1A in tomato is required for the β-oxidation stage of JA biosynthesis. The involvement of β-oxidation in JA biosynthesis implies an important role for peroxisomes in orchestrating plant responses to this stress hormone. A remarkable feature of plant peroxisomes is their capacity to alter their enzymatic content in response to environmental or developmental cues (Olsen, 1998; Corpas et al., 2001). The induced expression of genes involved in β-oxidation and peroxisome biogenesis in response to wounding and pathogen infection further suggests that biotic stress may be a

trigger for peroxisome proliferation in certain plant tissues (Lopez-Huertas et al., 2000; Mysore et al., 2002; Schenk et al., 2003; Castillo et al., 2004). As noted by Strassner et al. (2002), this degree of metabolic plasticity raises the possibility that the terminal steps in JA biosynthesis occur in a specialized type of peroxisome. Other results from studies with *acx1* are consistent with this idea and further suggest that JA-producing peroxisomes play an important role in the generation of signals that mediate systemic defense responses to herbivore attack (Li et al., 2005). Recent studies have shown that plastid enzymes of the octadecanoid pathway are spatially restricted to the companion cell-sieve tube element complex of the vascular bundle (Ryan, 2000; Hause et al., 2003; Howe, 2005). That wound-induced JA accumulation is also enriched in vascular tissues (Stenzel et al., 2003) would suggest that ACX1 and other peroxisomal enzymes of the pathway are located in these cell types as well. Support for this idea comes from studies showing that peroxisomes exist in sieve tube elements of root phloem (Jedd and Chua, 2002) and that soybean ACX1 is localized in the phloem cells of hypocotyl tissue (Agarwal et al., 2001). Future studies aimed at determining the location of JA-producing peroxisomes may provide additional insight into the role of oxylipins in long-distance wound signaling.

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Chapter 5

Acyl-CoA Oxidases that Function in the β-Oxidation Stage of Jasmonic Acid Biosynthesis are Essential for Insect Resistance and Pollen Development in *Arabidopsis*

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Abstract

The plant hormone jasmonic acid (JA) and its bioactive C18 precursors regulate a variety of developmental and defense-related processes. The final steps in the production of JA (a C12 cyclopentanone) require the action of peroxisomal β-oxidation enzymes that remove six carbon atoms from a C18 precursor. Among the enzymes involved in this metabolic transformation is acyl-CoA oxidase (ACX), which catalyzes the first step of the β-oxidation cycle. The purpose of this study was to identify *acx* mutants of *Arabidopsis* that are deficient in JA synthesis, and to use these mutants to investigate the role of the β-oxidation in jasmonate-signaled processes. We show that JA biosynthesis in *Arabidopsis* involves the so-called medium-to-long chain subfamily of ACXs that includes *ACX1* and *ACX5*. *acx1/5* double mutants were deficient in wound-induced JA accumulation and gene expression, and were compromised in resistance to the lepidopteran insect *Trichoplusia ni*. Unlike mutants that are blocked in JA perception or the production of C18 cyclopentenoids, *acx1/5* plants maintained strong resistance to the fungus *Alternaria brassicicola*. These results indicate that JA and its C18 precursors regulate defense responses against different types of biotic stress. The JA deficiency in *acx1/5* plants also resulted in reduced pollen viability and fecundity. Consistent with this finding, analysis of transgenic lines expressing *ACX1 promoter*-reporter fusions showed that *ACX1* is highly expressed in pollen, as well as other reproductive and vegetative tissues. We conclude that jasmonate signals derived from peroxisomal β-oxidation in *Arabidopsis* are essential for both male fertility and defense against leaf-eating insects.

Introduction

Plant responses to biotic stress are coordinated by a network of signal transduction pathways that control a wide range of physiological processes. Jasmonic acid (JA) and related members of the jasmonate family signaling compounds (collectively called JAs) play a central role in orchestrating these responses (Glazebrook, 2005; Halitschke and Baldwin, 2005; Howe, 2005). Although JAs are often regarded as stress signals, it is now clear that they also regulate a variety of developmental processes. Included among these are carbon/nitrogen partitioning (Creelman and Mullet, 1997), tendril coiling (Weiler et al., 1993), glandular trichome development (Li et al., 2004), root growth (Staswick et al., 1992), and various aspects of male and female reproductive function (Feys et al., 1994; McConn and Browse, 1996; Li et al., 2004). A current challenge in the field of jasmonate signaling is to understand the molecular mechanisms by which individual bioactive JAs regulate specific target processes.

The octadecanoid pathway for JA biosynthesis is initiated in the chloroplast and terminated in peroxisomes (Figure 5.1). Many of the enzymes and corresponding genes involved in the pathway have been identified (Schaller et al., 2005). A chloroplastic lipoxygenase initiates JA synthesis by adding molecular oxygen to linolenic acid (18:3). The resulting 13-hydroperoxy fatty acid is converted to 12-oxo-phytodienoic acid (OPDA) by the sequential action of allene oxide synthase (AOS) and allene oxide cyclase (AOC). Although little is known about the mechanism of plastid-to-peroxisome transport of octadecanoids, a peroxisomal ATP-binding cassette transporter was recently implicated in this process (Theodoulou et al., 2005). Within the peroxisome, OPDA

Figure 5.1 The octadecanoid pathway for JA biosynthesis.

Trienoic fatty acids (18:3 and 16:3) are converted within the chloroplast to 12-oxo-phytodienoic acid (OPDA) and dinor-OPDA, respectively. These cyclopentenone intermediates are transported to the peroxisome via a pathway that involves an ABC-transporter (CTS/PED3/PXA1), and then reduced by OPDA reductase (OPR3). The resulting cyclopentanone compounds (OPC8 and OPC6) are ligated to CoA by an acyl-CoA synthetase (ACS). Successive rounds of β-oxidation yield JA. See text for details. The three core enzymes in the β-oxidation cascade are acyl-CoA oxidase (ACX), multifunctional protein (MFP), and 3-ketoacyl-CoA thiolase (KAT). LOX, lipoxygenase; AOS, allene oxide synthase; AOC, allene oxide cyclase; OPC8, 3-oxo-2-(2'-pentenyl)-cyclopentane-1-octanoic acid; OPC6, 3-oxo-2-(2'-pentenyl)-cyclopentane-1-hexanoic acid; OPC4, 3-oxo-2-(2'-penetenyl)-cyclopentane-1-butyric acid; JA, (+)-7-iso-jasmonic acid.

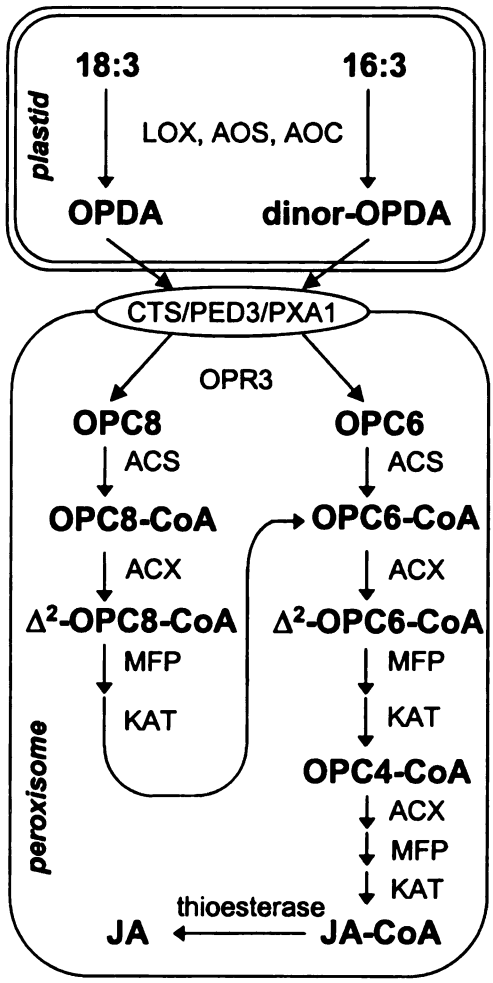

Figure 5.1 The octadecanoid pathway for JA biosynthesis.

reductase (OPR3) converts OPDA to cyclopentanone 3-oxo-2(2'[Z]-pentenyl)-cyclopentane-1-octanoic acid (OPC8). An acyl-CoA synthetase (ACS) is presumably required to produce OPC8-CoA, which subsequently enters the β-oxidation cycle that yields JA (Li et al., 2005). Peroxisomal enzymes related to 4-coumarate-CoA ligase were recently shown to catalyze the formation of OPDA-CoA and OPC8-CoA *in vitro* (Schneider et al., 2005). A parallel JA biosynthetic pathway starting from chloroplastic pools of hexadecatrienoic acid (16:3) has also been described (Weber et al., 1997) (Figure 5.1).

β-oxidative breakdown of fatty acids in plant cells occurs in peroxisomes. The three "core" enzymes involved in this pathway are acyl-CoA oxidase (ACX), a multi-functional protein (MFP) possessing 2-trans-enoyl-CoA hydratase and L-3-hydroxyacyl-CoA dehydrogenase activities and 3-keto-acyl-CoA thiolase (KAT) (Graham and Eastmond, 2002) (Figure 5.1). Seminal work by Vick and Zimmerman (Vick and Zimmerman, 1984) provided the first evidence that JA biosynthesis involves β-oxidation. Only recently, however, has specific enzymes been implicated in this stage of the pathway. The *ACX1A* gene product in tomato was shown to metabolize OPC8-CoA and to contribute to the vast majority of JA production in wounded leaves (Li et al., 2005). Genetic evidence also indicates that the *ACX1* and *KAT2* genes in *Arabidopsis* have a role in wound-induced JA production (Cruz Castillo et al., 2004; Pinfield-Wells et al., 2005). The persistence of significant levels of JA in *acx1* and *kat2* mutants, however, suggests that additional members of these gene families contribute to JA production in *Arabidopsis*.

Increasing evidence indicates that OPDA, JA, and certain JA derivatives promote different physiological responses (Mithofer et al., 2005; Schaller et al., 2005; Schilmiller and Howe, 2005). For example, JA is strictly required for male fertility in *Arabidopsis* (McConn and Browse, 1996; Stintzi and Browse, 2000). Conversely, OPDA rather than JA is thought to be the active signal for the tendril coiling response of *Bryonia* (Weiler et al., 1993). Studies of the *Arabidopsis opr3* mutant have provided strong evidence that OPDA promotes jasmonate-based resistance to *Bradysia impatiens* and *Alternaria brassicicola* in the absence of JA (Stintzi et al., 2001). On the other hand, JA biosynthesis is needed for production of the systemic wound signal in tomato, as well as for induced resistance of this species to lepidopteran attackers (Li et al., 2005). These results raise the possibility that plastid-derived OPDA and peroxisome-derived JA activate host defense responses to different biotic threats, and that the transformation of OPDA to JA is necessary for a subset of jasmonate-signaled defense responses.

A better understanding of the role of β-oxidation in jasmonate signaling would be facilitated by the identification of *Arabidopsis* mutants that fail to convert OPC8-CoA to JA. Although *Arabidopsis* mutants affected in the β-oxidation stage of JA synthesis have been reported (Cruz Castillo et al., 2004; Pinfield-Wells et al., 2005), none have been shown to exhibit physiological hallmarks of JA deficiency (e.g., male sterility). Here we report that simultaneous disruption of two *ACX* genes (*ACX1* and *ACX5*) effectively abolishes wound-induced JA accumulation in *Arabidopsis* leaves. We demonstrate that this defect compromises defense against the lepidopteran insect *Trichoplusia ni*, but does not impair jasmonate-mediated resistance to the fungus *Alternaria brassicicola*. These results support the hypothesis that JA and its C18 cyclopentenoid precursors promote

host resistance to different types of biotic stress. We also show that simultaneous loss of function of ACX1 and ACX5 depletes JA accumulation in flowers to a level below that required for normal pollen development and fecundity. Taken together, our findings show that the β-oxidation stage of JA biosynthesis is essential for both developmental and defense-related processes in *Arabidopsis*.

Materials and Methods

Plant material and growth conditions

Plants (*Arabidopsis* ecotype Columbia) were grown in soil in a growth chamber maintained under 16 h of light (100 $\mu E\ m^{-2}\ s^{-1}$) at 21°C and 8 h of dark at 21°C. T-DNA tagged lines from the SALK collection (Alonso et al., 2003) were obtained from the *Arabidopsis* Biological Resource Center (ABRC, Ohio State University). PCR assays were used to screen for plants that are homozygous for T-DNA insertions in *ACX1* (SALK_041464) and *ACX5* (SALK_009998) (Figure 5.2). These assays employed the LBb1 T-DNA oligonucleotide (http://signal.salk.edu/tdna_FAQs.html) and appropriate gene-specific primers (*acx1*, 64L 5'-GCAGACAGGAAGAATTTGTGAGAGTT TGG-3' and 64R 5'-GTGGTGGACATGGATACTTGTGGTG-3'; *acx5*, 98L 5'-CCGA GTCATTGAGTGGATCCT-3' and 98R 5'-CTGGAAAGGCTCCTTCTGGGA-3'). The T-DNA insertion locations were confirmed by DNA sequencing of the amplified PCR products. The *acx1/5* double mutant was generated by crossing the single mutants, and screening the resulting F_2 progeny for plants exhibiting reduced seed set. The identity of

Figure 5.2 Identification of SALK lines harboring T-DNA insertions in *ACX1* and *ACX5*.

(A) Schematic diagram of the genomic organization of *ACX1* and *ACX5*. Introns and exons are depicted as block boxes and horizontal lines, respectively. The location of the T-DNA insertion (inverted triangle) within exon 10 of each gene, as well as the location of primers (arrows) that were used for PCR-based genotyping, is shown.

(B) PCR assays were used to confirm the homozygosity of T-DNA insertions in *ACX1* and *ACX5*. Genomic DNA isolated from WT, *acx1*, *acx5*, and *acx1/5* plants was used as a template for PCR reactions employing primer sets (see above) that flank the T-DNA insertion site, or primer sets that included a gene-specific primer and a T-DNA left-border primer. Lanes labeled "1": primer pair (64L and 64R) that flanks the T-DNA insertion in *ACX1*; lanes labeled "5": primer (98L and 98R) that flanks the T-DNA insertion in ACX5; lanes labeled "T1": *ACX1* gene-specific primer (64L) and a T-DNA left-border primer (LBb1); lanes labeled "T5": *ACX5* gene-specific primer (98R) and T-DNA left border primer (LBb1). The sizes (in kilobase pairs) of DNA markers (M) are indicated on far left.

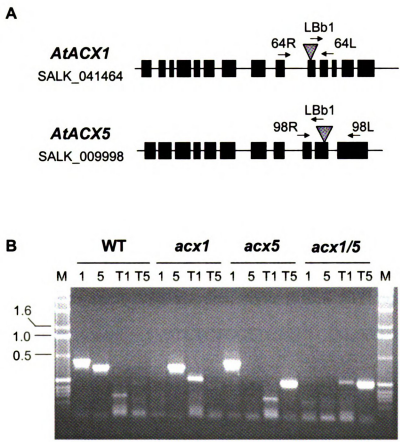

Figure 5.2 Identification of SALK lines harboring T-DNA insertions in *ACX1* and *ACX5*.

the double knockout was confirmed by PCR (Figure 5.2B). Prolonged exposures of *ACX1*-probed northern blots showed that low levels of *ACX1*-related transcripts accumulate in *acx1* leaves. RT-PCR analysis with primers that hybridize to the 5' untranslated region (5'-CACACTCGAGAATCTGAGACAATAG-3') of *ACX1* and the 3' end of the *ACX1* open reading frame (5'-GGGTCGACTCAGAGCCTAGCGGTACGAAG-3') detected full length *ACX1* transcripts in RNA isolated from WT but not *acx1* leaves (data not shown). DNA sequencing of RT-PCR products from *acx1* revealed that the low abundance transcripts are derived from transcriptional read-through of *ACX1* through the T-DNA insertion followed by splicing out the exon containing the T-DNA (data not shown). The corresponding ACX1 proteins are predicted to lack amino acids encoded by at least one *ACX1* exon, and thus can be assumed to be non-functional. PCR screening was also used to identify a homozygous *aos* T-DNA insertion mutant (SALK_017756). Primers used for these reactions were 5'-TTCTCTCCTTCTTCTCCGACG-3' and 5'-GATCCATCGGAGCCTAAACAC-3' (data not shown).

Western blot analysis of ACX protein levels

Recombinant His-tagged LeACX1A was affinity purified as previously described (Li et al., 2005). Rabbit polyclonal antibodies against this antigen were produced by a commercial vendor (Cocalico Biologicals, Reamstown, PA) according to their standard protocol. Western blot analysis was performed with 30 μg of total protein extracted from three-week old rosette leaves. Proteins were separated on 10% SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore, Bedford, MA) according to

standard procedures (Harlowe and Lane, 1988). Membranes were incubated at 24°C for 1 h with anti-LeACX1A antibodies that were diluted 1:1000 in TTBS (Tris-buffered saline with 0.1% Tween 20) containing 1% nonfat milk. As a control, duplicate protein blots were incubated with pre-immune serum obtained from the same rabbit that was immunized with LeACX1A. Blots were washed 3 times with TTBS and then incubated with a peroxidase-conjugated anti-rabbit secondary antibody (1:25,000 dilution; Sigma, St. Louis, MO). ACX protein-antibody complexes were visualized with the SuperSignal West Pico Chemiluminescent substrate (Pierce, Rockford, IL) according the manufacturers instructions.

JA measurements

Four-week old plants were wounded twice with a hemostat across the mid-vein of each rosette leaf. At various times after wounding, leaf tissue (200-300 mg) from at least three different plants of the same genotype was pooled and frozen in liquid nitrogen. Tissue was also collected from unwounded control plants grown in the same flat. JA was extracted according to the vapor-phase extraction procedure (Schmelz et al., 2004), and quantified by GC-MS as previously described (Li et al., 2005). For JA measurements in flowers, unopened buds and the first two opened flowers within the flower cluster were pooled from at least five inflorescences of each genotype. A total of 50 to 60 mg of flower tissue was used for each JA extraction.

RNA blot analysis

Wounding and harvesting of leaf tissue was done as described above. RNA extraction and gel blot analysis was performed as previously described (Li et al., 2002). Probes prepared from full-length cDNA clones for *VSP-1* (stock# 114D3), *OPR3* (U13428), and *ACX1* (U14146) were obtained from the ABRC. RNA quality and equal loading was confirmed by staining duplicate gels with ethidium bromide, as well as by hybridization of blots to a cDNA probe for *Actin-8*. This cDNA was obtained by RT-PCR with the following primers: 5'-GARAARATGACNCARATNATGTTYGARACNTT-3' and 5'-TCYTTNCTNATRTCNACRTCRCAYTTCATDAT-3'.

Insect feeding trials and fungal pathogenicity assays

Trichoplusia ni (cabbage looper) eggs were obtained from Benzon Research (Carlisle, PA) and hatched at 30°C. Within 8 h of hatching, a single larva was transferred to a four-week-old host plant. Feeding trials were conducted over a period of 10 days in a growth chamber maintained under 12 h of light (100 $\mu E/m^2/s$) at 21°C and 12 h of dark at 21°C. At the end of the trial, individual live larvae were weighed and the plants were photographed. In trials involving JA-treated plants, 20 µl of a solution containing 1 mM JA (Sigma) and 0.1% Tween 20 was applied to each rosette leaf immediately before insect challenge.

Alternaria brassicicola (strain MUCL20297) was grown on potato dextrose agar at 25°C for 10 days, at which time conidia/conidiospores were collected in water. Four-week-old soil-grown *Arabidopsis* plants were inoculated on the leaf surface by applying a 5 µl drop of a suspension containing $5x10^5$ spores ml^{-1}. Flats containing the inoculated

plants were covered with a plastic transparent cover to maintain high humidity. Plants were grown under standard conditions for 5 days prior to assessing the disease phenotype.

Pollen viability measurements

Pollen viability was measured by double staining of pollen grains with fluorescein diacetate (FDA) and propidium iodide (PI). The procedure was essentially as described by McConn and Browse (McConn and Browse, 1996), with minor modifications. Two mg ml^{-1} FDA in acetone was added drop wise to a 20% (w/v) sucrose solution. Pollen from newly-dehisced anthers was transferred to a glass slide. Following the addition of equal volumes of FDA and PI solution (1 μg ml^{-1}) to the pollen, the sample was covered with a cover slip and incubated in the dark for ~10 min. Pollen was visualized under UV illumination with an epifluorescence microscope (Zeiss Axiophot, Germany) equipped with a DAPI filter set (excitation at 365 nm; emission longpass at 450 nm). FDA is de-esterified within living cells to fluorescein, which emits a green fluorescence signal under UV excitation. Only non-viable cells incorporate PI, which fluoresces red-orange under UV light. *In vitro* pollen germination was done as previously described (Thorsness et al., 1993)

Results

ACX1 and ACX5 catalyze wound-induced JA biosynthesis in Arabidopsis leaves

The persistence of significant levels of JA in *acx1* mutants of *Arabidopsis* suggested that additional ACX isoforms likely contribute to production of the hormone (Cruz Castillo et al., 2004; Pinfield-Wells et al., 2005). A good candidate for such an enzyme is ACX5, which is highly related to both ACX1 and the ACX1A isoform that catalyzes the vast majority JA biosynthesis in tomato leaves (Figure 5.3A). Our approach for testing this hypothesis was to isolate and characterize T-DNA knockout mutants that are defective in ACX1 *(acx1* plants), ACX5 (*acx5* plants), or both ACX1 and ACX5 (*acx1/5* plants) (see Methods). RT-PCR experiments showed that the *acx1* and *acx5* mutants used in this study fail to express functional *ACX1* and *ACX5* transcripts, respectively, indicating that we were assessing the null phenotypes (data not shown).

To determine whether the various *acx* mutants are affected in the expression of ACX isoforms that have a putative role in JA biosynthesis, we performed immunoblot assays with a polyclonal antibody raised against LeACX1A. The antiserum reacted strongly and specifically with a protein in wild-type (WT) leaves (Figure 5.3B). The apparent molecular weight of this protein was in good agreement with the calculated molecular weight of ACX1/5 (~74,300). That this polypeptide was not detected in *acx1* leaves established its identity as ACX1. These results do not exclude the possibility that the antibody cross-reacts with other ACX isoforms whose accumulation is below the level of detection. This interpretation is consistent with the finding that *acx5* leaves accumulate WT levels of the LeACX1A-related protein, whereas *acx1/5* leaves do not

Figure 5.3 The *Arabidopsis* ACX1 and tomato ACX1A isoforms are immunologically related.

(A) Phylogenetic analysis of the *Arabidopsis* ACX family. A neighbor-joining phylogeny was constructed in PAUP4.0* from the deduced amino acid sequences of the *Arabidopsis* ACX family members (AtACX1-AtACX6). Also included in the phylogeny is the tomato ACX1A isoform that has an established role in JA biosynthesis. Numbers indicate percent bootstrap support for each branch of the phylogeny.

(B) Western blot analysis of ACX protein levels in WT and *acx* knockout plants. Protein from three-week old rosettes of the indicated genotype was blotted and probed with a polyclonal antibody against tomato ACX1A (LeACX1A; left panel) or an equivalent amount of pre-immune serum (right panel). The arrow indicates an *Arabidopsis* ACX protein that specifically cross-reacts with the immune serum. Molecular weight standards (kDa) are indicated on the left side of each blot.

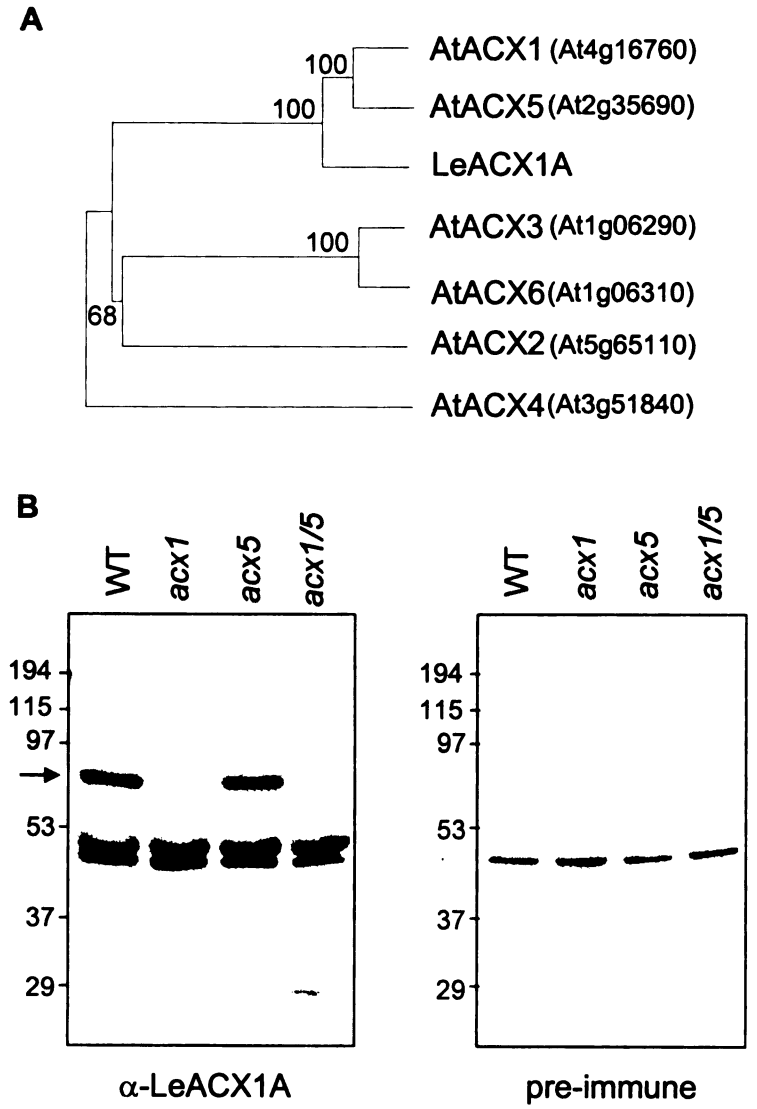

Figure 5.3 The *Arabidopsis* ACX1 and tomato ACX1A isoforms are immunologically related.

(Figure 5.3B). These results demonstrate that AtACX1 and LeACX1A are immunologically related, and that ACX1 does not accumulate in either *acx1* or *acx1/5* leaves.

To determine the relative contribution of ACX1 and ACX5 to JA synthesis, we used gas chromatography-mass spectrometry to measure the endogenous levels of JA in unwounded (control) and wounded leaves (Figure 5.4A). The basal level of JA in unwounded *acx1* and *acx1/5* plants (39 ± 11 and 50 ± 37 pmol/gfw, respectively) was reduced approximately two-fold in comparison to JA levels in WT and *acx5* plants (95 ± 31 and 83 ± 23 pmol/gfw, respectively). Mechanical wounding of WT leaves caused a sharp increase in JA accumulation. Wound-induced JA levels in *acx1* leaves were reduced to 20.6% of WT levels, which is consistent with the results of previous studies (Cruz Castillo et al., 2004; Pinfield-Wells et al., 2005). JA accumulation in wounded *acx5* plants slightly exceeded (by approximately 1.5-fold) that in WT plants. In contrast to the relatively large (>25-fold) wound-induced increase in JA levels in WT, *acx1*, and *acx5* plants, JA levels in the *acx1/5* mutant did not increase significantly in response to wounding. We estimated that the total amount of JA in wounded *acx1/5* leaves was ~1% of WT levels.

RNA blot analysis was used to determine the effect of the *acx* mutations on the expression of two wound-responsive genes, *OPR3* and *VSP-1* (Berger et al., 1995; Mussig et al., 2000). Mechanical wounding activated the expression of both genes in WT, *acx1*, and *acx5* leaves (Figure 5.4B). The most notable difference between these three genotypes was that, at the 12 h time point, *OPR3* expression in *acx5* leaves was considerably higher than that in WT and *acx1* plants. Wound-induced expression of both

Figure 5.4 Effect of *acx* mutations on wound-induced JA accumulation and gene expression.

(A) JA levels in *acx* knockouts in response to mechanical wounding. Leaves on four-week old plants of the indicated genotype were wounded twice across the mid-vein. At various times after wounding (1.5 or 3 h), tissue was collected for JA extraction and quantification by GC-MS. Unwounded (UW) leaf tissue was collected as a control. Values represent the mean and SD for three independent JA extractions per genotype.

(B) Time-course expression of wound-induced genes. Leaves were wounded as described above. At the indicated times after wounding, leaf tissue was harvested for RNA extraction. Leaf tissue from unwounded control plants was used for the "0" h time point. RNA blots were hybridized to ^{32}P-labelled cDNA probes for *OPR3*, *VSP-1*, and *ACX1*. Blots were also hybridized to a probe for *Actin-8* (*ACT-8*) as a loading control.

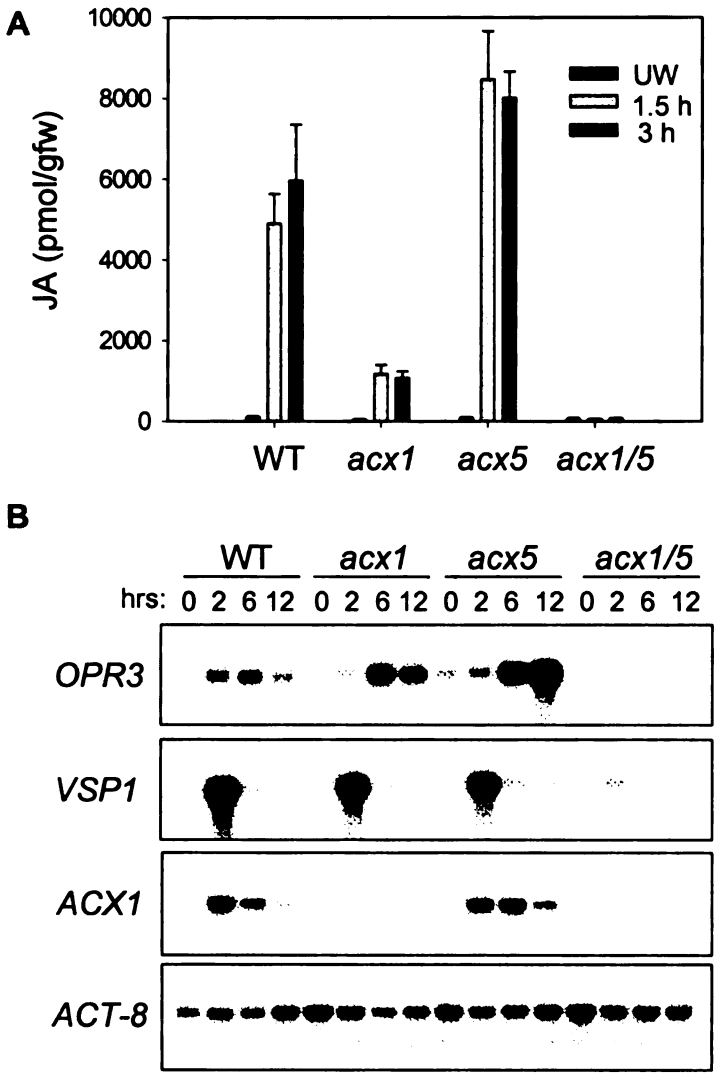

Figure 5.4 Effect of *acx* mutations on wound-induced JA accumulation and gene expression.

VSP-1 and *OPR3* was severely diminished in *acx1/5* leaves; expression of these genes in the *acx1/5* background was only detected upon prolonged exposure of autoradiographs. As expected, expression of *ACX1* was detected in wounded WT and *acx5* plants, but not in wounded *acx1* and *acx1/5* mutants. These results indicate that ACX1/5-mediated JA production is required for the expression of wound-responsive genes.

acx1/5 plants are more susceptible to Trichoplusia ni feeding but maintain resistance to infection by Alternaria brassicicola

To better define the role of the β-oxidation stage of JA biosynthesis in jasmonate-based plant defense against insect attack, WT and *acx* mutant plants were challenged with *Trichoplusia ni* larvae (cabbage looper worm). In three independent feeding trials, *acx1/5* plants reproducibly suffered more damage than WT, *acx1*, or *acx5* plants (Figure 5.5A). Consistent with this observation, the average weight of larvae reared on *acx1/5* plants was significantly greater than that of larvae grown on the other host genotypes (Figure 5.5B). Treatment of *acx1/5* plants with JA immediately before the start of the feeding trail was sufficient to restore resistance of the mutant to *T. ni* attack (data not shown). Moreover, larval weight measurements showed the *T. ni* performance on JA-treated *acx1/5* plants was not significantly different ($P = 0.25$) from that on untreated WT plants (Figure 5.5B). These results demonstrate that ACX1/5 function is essential for resistance of *Arabidopsis* to attack by *T. ni*.

Pathogenicity assays were performed to determine whether *acx1/5* plants are altered in their resistance to *Alternaria brassicicola* infection. This necrotrophic fungal pathogen was previously shown to activate jasmonate-dependent defense responses in

Figure 5.5 *acx1/5* plants are susceptible to attack by *Trichoplusia ni*.

(A) Four-week-old WT and *acx* mutant plants were challenged with newly-hatched *Trichoplusia ni* larvae (one 1st-instar larva per plant). Plants were photographed 10 d after the start of the feeding trial.

(B) The average weight of larvae reared on each host genotype was determined at the end of the feeding trial. In this experiment, larvae were allowed to move freely between *acx1* and *acx5* plants. Thus, larval weight data for caterpillars recovered from these two genotypes were combined. Additional experiments showed that larval performance on *acx1* and *acx5* plants was not significantly different (data not shown). Also shown is larval weight data for *acx1/5* plants that were treated with JA prior to initiation of the feeding trial (*acx1/5* + JA). Data show the mean and SD of the following number of larvae: WT, 40; *acx1* + *acx5*, 42; *acx1/5*, 39; *acx1/5* + JA, 8. The asterisk indicates that the weight of larvae grown on *acx1/5* plants was significantly greater ($P < 0.001$; Mann-Whitney Rank Sum Test) than that of larvae reared on WT plants.

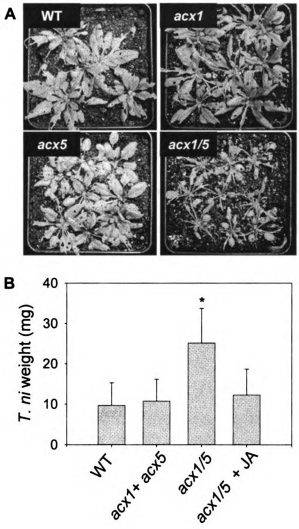

Figure 5.5 *acx1/5* plants are susceptible to attack by *Trichoplusia ni*.

Arabidopsis (Penninckx et al., 1996; Thomma et al., 1998; Stintzi et al., 2001). WT and *acx1/5* rosette leaves were inoculated with a suspension of *A. brassicicola* spores. The *coi1-1* mutant that is susceptible to *A. brassicicola* (Thomma et al., 1998) was also inoculated as an additional control. At 3 days post-inoculation (dpi), both WT and *acx1/5* plants developed a typical resistance response, manifested by the formation of brown necrotic lesions at the site of spore inoculation (Figure 5.6A and B). In contrast, *coi1* leaves were heavily colonized by the pathogen (Figure 5.6C and E). This susceptibility phenotype was also observed in a mutant that harbors a T-DNA insertion in the *AOS* gene that encodes a plastidic enzyme of the octadecanoid pathway (Figure 5.6D and F). The most straightforward interpretation of these results in that resistance of *Arabidopsis* to *A. brassicicola* does not depend on the β-oxidation stage of JA biosynthesis, but rather requires the synthesis of an AOS-derived C18 signal that works through COI1, as previously proposed (Stintzi et al., 2001).

acx1/5 plants are impaired in male fertility

Severe deficiencies in JA biosynthesis or perception in *Arabidopsis* result in male sterility (Devoto and Turner, 2005). During the growth of F_2 plants derived from a cross between *acx1* and *acx5*, we observed that the *acx1/5* double mutant produced very few seed-containing siliques and very few viable seeds. Both the *acx1* and *acx5* single knockout lines produced normal amounts of seed (data not shown). The *acx1/5*-mediated decrease in fecundity was quantified in a controlled experiment in which WT and *acx1/5* plants were grown side-by-side under identical conditions (Table 5.1). Treatment of

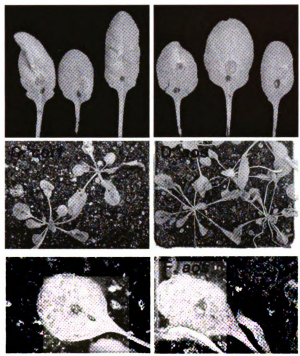

Figure 5.6 *acx1/5* plants maintain resistance to *Alternaria brassicicola*.

Four-week-old plants of the indicated genotype were inoculated with *Alternaria brassicicola* by applying a 5 ml drop of a spore suspension (5 x 10^5 spores ml^{-1}) on the leaf surface. Plants were photographed 5 days after inoculation. Panels E and F show a close-up view of leaves depicted in panels C and D, respectively.

Table 5.1. Reduction of seed-containing siliques in *acx1/5*

	WT	*acx1/5*	
siliques / plant	115.1 ± 25.8	105 ± 34.3	p = 0.399
siliques / plant with seed	83.8 ± 26.1	14.6 ± 9.3	p < 0.001
% siliques with seed	71.9 ± 10.1	13.1 ± 6.5	p < 0.001

The total number of siliques per plant as well as seed-containing siliques per plant were quantified for 14 wild-type and 32 *acx1/5* plants that were fully senesced and the percentage of siliques per plant containing seed was calculated. Values for wild-type (WT) and *acx1/5* represent the mean ± SD and statistical significance was determined with the Student's *t* test.

acx1/5 flowers with MeJA restored silique development and viable seed production (Figure 5.7A). Seed collected from MeJA-treated *acx1/5* plants did not require sucrose for germination or seedling establishment (data not shown).

Male sterility in JA-deficient *Arabidopsis* mutants is caused by a combination of defects in pollen viability, anther elongation, and anther dehiscence (McConn and Browse, 1996; Stintzi and Browse, 2000). However, *acx1/5* flowers did not exhibit obvious abnormalities in the timing of anther dehiscence or anther elongation (data not shown). We thus employed fluorescein diacetate-propidium iodide staining to compare the viability of WT and *acx* pollen. In four independent experiments, pollen collected from WT, *acx1*, and *acx5* flowers exhibited approximately 80% viability (Figure 5.7B). In contrast, only about 30% of the pollen collected from *acx1/5* plants was viable. Decreased pollen viability in *acx1/5* plants was correlated with a steep decline in JA levels in flower buds ($P<0.001$, Student's *t* test; Figure 5.7C). These results indicate that the JA deficiency caused by *acx1/5* severely reduces plant fecundity, mainly as a result of a defect in pollen development.

Figure 5.7 *acx1/5* plants are defective in JA-mediated pollen development.

(A) Photograph of an *acx1/5* inflorescence. In the absence of JA treatment, the majority of siliques fail to develop and produced no viable seed. Treatment of stage-12 flowers with JA restored silique development (arrows) and viable seed production.

(B) Pollen from newly-dehisced flowers was collected and double-stained with fluorescein diacetate and propidium iodide to determine percent viability. All measurements were performed in quadruplicate, with between 100 and 500 pollen/genotype. The asterisk indicates that pollen viability in *acx1/5* plants was significantly less than that in WT plants ($P = 0.002$, Mann-Whitney Rank Sum Test).

(C) JA levels are reduced in *acx1/5* flowers. Data show the mean and SD of three independent experiments. Experiment involved at least three JA extractions from independent pools of similarly-staged flowers.

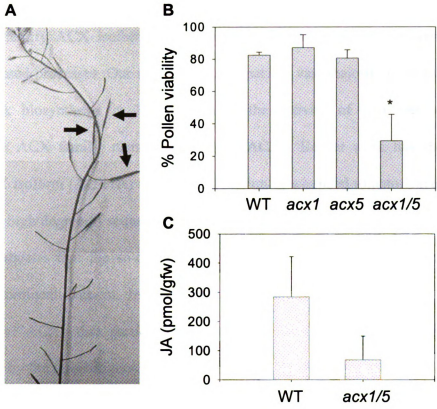

Figure 5.7 *acx1/5* plants are defective in JA-mediated pollen development.

Discussion

Arabidopsis provides an excellent model system in which to study the role of jasmonates in the plant life cycle (Gfeller and Farmer, 2004; Devoto and Turner, 2005). In this study, we took advantage of powerful genetic tools in *Arabidopsis* (Alonso et al., 2003) to identify ACX isoforms that contribute to JA-dependent developmental and defense-related processes. Our results indicate that the vast majority (>98%) of wound-induced JA biosynthesis in leaves requires the activity of two members of the *Arabidopsis* ACX family, namely ACX1 and ACX5. Recent work has shown that a single ACX isoform (ACX1A) fulfills this function in wounded tomato leaves (Li et al., 2005). The high degree of sequence similarity between *Arabidopsis* ACX1/5 and tomato ACX1A indicates that the so-called medium-to-long chain subfamily of plant ACXs plays a prominent role in JA biosynthesis. This information should facilitate the discovery of ACXs that participate in JA biosynthesis in other plant species. As determined by the three-dimensional structure of ACX1 (Pedersen and Henriksen, 2005), the wide fatty acyl-binding pocket of these enzymes is consistent with their ability to metabolize a broad range of straight-chain acyl-CoAs, and cyclopentenoid-CoA precursors of JA as well (Li et al., 2005).

Based on the analysis of *acx1* and *acx5* single mutants, it is evident that the contribution of ACX1 to wound-induced JA production is significantly greater than that of ACX5. Our finding that wounded *acx1* leaves accumulate ~20% of WT levels of JA is in agreement with other studies on ACX1 (Cruz Castillo et al., 2004; Pinfield-Wells et al., 2005). Despite the major contribution of this isoform to JA production, loss of ACX1

function did not significantly reduce the expression of wound-responsive genes that are regulated by the JA signaling pathway (Figure 5.4B). We propose that the amount of JA produced in *acx1* plants is sufficient to activate subsequent signal transduction events that lead to gene expression, and that other ACXs contribute to JA-mediated defense responses in *Arabidopsis*.

Introduction of an *acx5* null mutation into the *acx1* background nearly abolished wound-induced JA accumulation and the expression of defense-related genes, indicating that ACX5 plays a role in wound-induced expression of JA-responsive genes. Paradoxically, however, JA accumulation in wounded *acx5* leaves was not impaired, but rather was slightly greater than that in WT. This result is in keeping with the idea that ACX5 makes a relatively minor contribution to JA production, and that ACX1 activity in WT leaves is not limiting for JA biosynthesis. Although *ACX5* is expressed in leaves, northern blot analysis and quantitative RT-PCR experiments showed that its expression level is low in comparison to *ACX1* (ALS and GAH, unpublished data)(Kamada et al., 2003). That wound-induced JA levels in *acx5* plants were slightly greater than in WT plants raises the possibility that ACX5 may actually impede JA biosynthesis in the presence of ACX1. For example, it is possible that ACX5 (or ACX1/5 heterodimers) is less efficient than ACX1 in the metabolism of OPC8-CoA. In *acx5* leaves, ACX1 would not compete with the less effective enzyme for substrate, thus allowing a higher flux through the β-oxidation pathway. Comparison of the *in vitro* kinetic parameters of ACX1 and ACX5 will be useful to test this idea.

The reduced fecundity of *acx1/5* plants demonstrates that β-oxidation plays a critical role in male reproductive development in *Arabidopsis*. The *Arabidopsis aim1*

mutant that is defective in the MFP-catalyzed step of β-oxidation also exhibits severely reduced fertility (Richmond and Bleecker, 1999). However, it has not yet been determined whether *aim1*-mediated sterility is caused by decreased JA production. The ability of exogenous JA to restore fertility to *acx1/5* plants, as well as the reduced levels of JA in *acx1/5* flowers, demonstrates that the sterile phenotype of this mutant results from a block in the β-oxidative stage of JA biosynthesis. Thus, our results confirm and extend previous studies showing that JA is strictly required for male fertility in *Arabidopsis*.

It is noteworthy that *acx1/5* flowers do not exhibit obvious defects in anther elongation or pollen dehiscence that occur in many other JA-deficient mutants (e.g., *opr3*). Rather, the *acx1/5* metabolic block appears to impair male fertility mainly by decreasing pollen viability. Support for this hypothesis comes from the finding that *ACX1* expression in the stamen is much more prominent in the pollen than it is in the anther or filament (AJKK and GAH, unpublished results). Additional ACX family members that are expressed in stamens may produce enough JA to satisfy these aspects of JA-dependent anther development. The absence of anther elongation and dehiscence phenotypes in *acx1/5* flowers may explain the ability of the mutant to produce limited amounts of viable seed under some growth conditions. We thus suggest that the amount of JA in *acx1/5* flowers (~25% of WT levels) is close to the threshold level that is needed for normal reproductive vigor.

Peroxisomal β-oxidation plays a central role in the catabolism of storage lipids during seedling establishment. Several mutations that disrupt β-oxidation have been shown to arrest seedling development in the absence of exogenous sucrose (Hayashi et

al., 1998; Zolman et al., 2001). The demonstration that *acx1* and *acx5* single mutants, as well as the *acx1/5* double mutant, germinate and grow in the absence of sucrose indicates that these isoforms are not absolutely required for normal seedling establishment (Adham et al., 2005; ALS and GAH, unpublished results). Increasing evidence, however, indicates that ACX1 does in fact participate in the mobilization of seed storage lipids. First, ACX1 acts on a broad range of medium- and long-chain fatty acyl-CoAs *in vitro* (Hooks et al., 1999). Second, enzyme extracts from *acx1* mutant seedlings show a striking deficiency in the metabolism of long-chain acyl-CoAs (Adham et al., 2005). Third, introduction of the *acx1* mutation into a genetic background that lacks ACX2, which also metabolizes long chain acyl-CoAs (Hooks et al., 1999), results in a sucrose-dependent growth phenotype (Adham et al., 2005; Pinfield-Wells et al., 2005). These biochemical and genetic data are further supported by our finding that *ACX1* is highly expressed in germinating seedlings (AJKK and GAH, unpublished results). Thus, it can be proposed that ACX1 serves a dual function in bulk fatty acid catabolism during seedling development and in JA biosynthesis. Direct support for this idea comes from the finding that the orthologous tomato enzyme (ACX1A) metabolizes both straight-chain acyl-CoAs and cyclopentenoid-CoA precursors of JA (Li et al., 2005).

A striking phenotype of *acx1/5* plants is their susceptibility to herbivore attack. The severe JA deficiency in wounded *acx1/5* leaves, together with the ability of exogenous JA to restore protection to herbivory (Figure 5.5B), indicates that JA performs an essential role in defense of *Arabidopsis* to *T. ni* attack. Because *acx1/5* plants fail to convert OPC8-CoA to JA, this finding excludes the possibility that resistance to this herbivore is mediated by a C18 jasmonate signal. We also have observed that *acx1/5*

plants have significantly reduced resistance to western flower thrips (*Frankliniella occidentalis*) (ALS and GAH, unpublished results), a cell content-feeding herbivore that activates the JA signaling pathway (Li et al., 2002; De Vos et al., 2005). The increased susceptibility of *acx1/5* plants to multiple leaf-eating insects is consistent with studies showing that JA is required for defense responses of tomato to attack by *Manduca sexta* larvae (Li et al., 2005). We thus conclude that the β-oxidation stage of JA biosynthesis is an essential component of the plant immune system, and that JA (or its derivatives) is the active signal for induced resistance to at least some types of phytophagous insects. Analysis of COI1-dependent defense responses in different JA biosynthetic mutants of *Arabidopsis* provides a powerful approach to determine the contribution of specific jasmonate signals to host resistance. In applying this approach to the *Arabidopsis opr3* mutant, Stintzi and associates proposed that OPDA acts as a signal for defense responses against *A. brassicicola* and the saprophagous insect *B. impatiens* (Stintzi et al., 2001). Our finding that *acx1/5* plants maintain resistance to *A. brassicicola*, whereas *coi1* and *aos* mutants do not, provides strong support for this idea. However, the increased susceptibility of *acx1/5* plants to *T. ni* feeding allows us to further define the role of OPDA in defense signaling. Specifically, our work with *Arabidopsis* and tomato (Li et al., 2005) *acx* mutants indicates that OPDA (or other C18 signals) is not sufficient to promote the full range of COI1-dependent defense responses. Rather, oxylipin-mediated resistance to many herbivores depends on the ability of the host plant to convert OPDA to JA via the β-oxidation pathway. This discovery distinguishes plants from their animal counterparts in which β-oxidation plays a primary role in the inactivation of oxylipin signals derived from arachidonic acid. Future studies aimed at understanding how plastid-

derived OPDA and peroxisome-derived JA regulate resistance to specific plant attackers are clearly warranted.

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Chapter 6

Conclusions and Future Directions

At the beginning of this dissertation research, the identification of genes encoding CYP74 cytochromes P450 was picking up pace. The goals of chapters 2 and 3 involved studying the CYP74 family in tomato to better understand oxylipin biosynthesis in this plant. Comparatively, tomato is a rich source of CYP74 genes, whereas the model plant *Arabidopsis thaliana* contains only two CYP74 sequences in its genome. Identification and characterization of the first AOS that functions in the 9-LOX pathway has laid the groundwork towards understanding the function of the 9-AOS pathway in plants. There still remains the question of why some plants, such as *Arabidopsis*, lack a *9-AOS* gene but contain multiple copies of *9-LOX* genes. In all plants for which there is sequence information, *9-LOX*s can be identified. Likewise, all plants are believed to synthesize JA, which requires the presence of a *13-AOS* gene. It seems as if the 9-AOS pathway evolved from the *13-AOS*s with some plants recruiting *AOS* genes for transformation of 9-LOX products, while others have not. Alternatively, plants lacking the 9-AOS pathway may have lost their *9-AOS* genes. Regardless of how the 9-AOS pathway has evolved, we still don't understand the function or how plants benefit from having this pathway.

Future work for studying the 9-AOS pathway in tomato includes taking advantage of reverse genetics approaches (i.e. antisense or RNAi suppression, overexpression) to manipulate *9-AOS* expression levels. Some of this work has been started with transgenic plants suppressing or overexpressing *LeAOS3* already generated. While no obvious phenotypes were identified in primary transformants, generating homozygous lines for further testing is the next step. With these plants, we can determine whether suppression of *LeAOS3* expression leads to any developmental phenotypes, particularly in growing root tips where *LeAOS3* is primarily expressed. Antisense plants will also be useful for

testing for susceptibility to tomato root pathogens such as *Pythium* and *Fusarium* or to root knot nematodes. Additionally, plants ectopically expressing *LeAOS3* may exhibit phenotypic changes, which could give clues for the function of *LeAOS3*. One possibility we plan to test is whether the 9-LOX (and 9-AOS) pathway has a role in regulating JA biosynthesis. The induction of 9-LOX and 9-AOS activities by JA may function as a way to shift free linolenic acid away from JA production. If this is true we would expect to see higher levels and/or prolonged production of JA in wounded roots of antisense *LeAOS3* plants. Generation and testing of hypotheses like this will hopefully shed light on the physiological function of the 9-AOS pathway in plants.

While the preliminary data from microarray analysis of MeJA-regulated gene expression in tomato roots was presented in chapter 3, much more analysis is yet to be done. There is currently little known about JA signaled responses in non-aerial tissues. The genes identified in this experiment represent a valuable data set for looking at pathways and processes in roots that are controlled by JA.

The other half of this dissertation focuses on the role of β-oxidation in JA biosynthesis. At the time of cloning *LeACX1A*, there were no genes encoding β-oxidation enzymes associated specifically with JA biosynthesis. Work in tomato and *Arabidopsis* has shown conclusively that medium-long chain ACXs are required for JA production. Using *acx* mutants demonstrated the requirement of the β-oxidation stage of JA biosynthesis in insect defense. A recent disocvery in JA signaling is that cyclic precursors of JA (namely OPDA) have signaling properties of their own, some of which are distinct from JA. The finding that *Arabidopsis acx1/5* mutants are susceptible to a chewing insect but resistant to a necrotrophic pathogen supports this idea. For future studies, the tomato

and *Arabidopsis acx* mutants will be useful tools for further dissecting the roles of individual cyclic JA precursors in signaling.